Costs and Benefits of a Stress Response on Postnatal Development in the Eurasian Kestrel (*Falco tinnunculus*)

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Claudia MÜLLER

von

Unterbözberg AG

Promotionskomitee

Prof. Dr. Lukas Keller (Vorsitz)

PD Dr. Lukas Jenni (Leiter der Dissertation)

Prof. Dr. Heinz-Ulrich Reyer

Zürich, 2009

Die vorliegende Arbeit wurde am 25. Mai 2009 von der Mathematisch-naturwissenschaftlichen Fakultät der Universität Zürich als Dissertation angenommen.

Promotionskomitee: Prof. Dr. Lukas Keller (Vorsitz)
PD Dr. Lukas Jenni (Leiter der Dissertation)
Prof. Dr. Heinz-Ulrich Reyer

Table of Contents

Summary

Summary

Free-living vertebrates react to unpredictable events threatening the homeostasis by an increase in glucocorticoids, corticosterone in birds. With its steering role in energy allocation from actual activities to maintenance, corticosterone supports an organism to get over threatening situations, but concurrently future prospects possibly deteriorate because of negative impacts on growth and development. The aim of this study was to investigate in altricial bird chicks during development (a) the extent of the negative impacts of elevated corticosterone levels on growth, development and survival, i.e. on how stress hormones shape the phenotype and (b) how the levels of baseline corticosterone and the increase in corticosterone as a response to an acute stressor are modulated with the situation of the chick, i.e. age, hatching order and body condition. Additionally, we considered whether a variation in corticosteroid-binding-globulin (CGB) modulates circulating free corticosterone. This study was done with free-living Eurasian kestrels *Falco tinnunculus*, a small raptor species which hunts predominantly on voles and shows hatching asynchrony.

In **chapter 1,** we examined the effects of a recently developed method of corticosterone administration. We tested the performance of self-degradable corticosterone pellets to artificially increase circulating corticosterone in nestling Eurasian kestrels and barn owls *Tyto alba.* We assessed potential interactions of elevated corticosterone levels with the corticosterone-binding-globulin and with the endogenous corticosterone response to handling. The pellets induced a peak-shaped elevation in circulating total corticosterone during 2 to 3 days which reached levels attained by an acute stressor. An increase in corticosterone as a response to handling and restraint was absent during and up to 8 days after implantation and had recovered 20 days after implantation, indicating that negative feedback regulated circulating corticosterone. During artificially elevated total corticosterone levels, corticosteroid-binding-globulin capacity was also increased, resulting in a less pronounced elevation in free corticosterone.

The effect of such an artificial elevation in corticosterone by pellets during postnatal development in kestrel nestlings on diverse growth parameters and morphology before fledging was examined in **chapter 2**. During the 2 to 3 days of elevated corticosterone levels in the middle of the nestling stage, growth of wing feathers was significantly reduced to 71%, hand and tarsus growth to 14% and 26% respectively and body mass increase stopped in corticosterone-treated nestlings as compared with a placebo group. During the following five days until day 21, wing feather growth was still significantly suppressed to 84%, while hand, tarsus and body-mass growth were back to normal. Furcular fat store increase was not affected by the corticosterone treatment. Before fledging, wing feather length was 10% shorter, hand and tarsus 5% and 4% shorter and body mass 8.5% lower in corticosterone-treated nestlings than in placebo-treated nestlings. Feather, bone and body mass growth were reduced to different degrees, indicating that corticosterone had a differential effect on different structures. This demonstrates that corticosterone is involved in the control of developmental plasticity.

The developmental hypothesis proposes that the adrenocortical response to stress during postnatal development in birds should not develop when the benefits of elevated corticosterone do not outweigh the deleterious effects on growth and development. In **chapter 3**, we investigated three predictions developed from this hypothesis in kestrel nestlings. First, the adrenocortical response to handling and human presence at the nest increased from day 10 to 21, during the time when nestlings developed a vigorous defence behaviour. Second, nestlings with adequate body energy stores (furcular fat stores) mounted a strong adrenocortical response to an acute stressor, while nestlings with low energy stores had a reduced response and avoided additional energy expenses. Baseline corticosterone levels were negatively related to body energy stores. Third, both baseline corticosterone levels and the adrenocortical response to handling were not related to hatching order, but predominantly determined by body condition. The pattern of decreasing corticosterone levels with hatching order found in ad libitum fed nestlings in the lab may be superimposed by variation in body condition in free-living birds. Since corticosteroid-bindig-globulin capacity did not vary with any of the parameters investigated, free corticosterone levels showed a similar pattern as total levels. The modulation of the adrenocortical response during postnatal development seems to be adaptive to avoid the negative effects of elevated corticosterone on the phenotype for life.

In **chapter 4** we investigated the consequences of hatching asynchrony from the perspective of the last-nestling, rather than of the parent. Explaining the occurrence of hatching asynchrony, the brood reduction hypothesis postulates that brood size is adaptively reduced under conditions of food scarcity through starvation death of the last-hatched nestling. We showed that the strategy of the last-hatched nestling varies with food availability (yearly fluctuations in prey abundance and short-term impairment of hunting success of adults by rain). Under good conditions (no rain, large furcular fat stores) last-hatched nestlings had lower baseline corticosterone levels and a higher wing feather growth rate than older siblings, which allows catch-up growth. Short-term food restriction (rain) resulted in higher baseline corticosterone levels in last-hatched nestlings than in older siblings. Furcular fat stores were lower in last-hatched nestlings compared with older siblings. Growth of wing feathers and body mass was reduced and mortality increased in last-hatched nestlings with high corticosterone levels. We conclude that last-hatched nestlings modulate corticosterone levels and growth allocation in a condition-dependent way to make the best of a bad situation.

In summary, this study on altricial nestlings shows that the stress hormone corticosterone plays an important regulatory role in the response to varying conditions and in shaping the phenotype. The increase in corticosterone to an acute stressor, however, is modulated and fine-tuned in response to age and body condition, probably to optimise the adrenocortical response with respect to the prevailing external and internal conditions.

Zusammenfassung

Zusammenfassung

Freilebende Wirbeltiere reagieren auf unvorhersehbare Ereignisse, die ihre Homöostase gefährden, mit einer erhöhten Ausschüttung von Glucocorticoiden, bei Vögeln vor allem Corticosteron. Corticosteron unterstützt den Organismus beim Überstehen von schwierigen Situationen. Da es die vorhandene Energie von speziellen Prozessen wie Wachstum und Entwicklung zu Gunsten der Grundfunktionen umlenkt, kann es dabei aber eventuell die zukünftigen Aussichten des Individuums gefährden. Das Ziel dieser Studie war, bei einer Nesthocker-Art während der Entwicklungszeit zu untersuchen, a) in welchem Ausmass erhöhte Corticosteronspiegel negative Auswirkungen auf Wachstum, Entwicklung und Überleben haben, d.h., wie Stresshormone den Phänotyp beeinflussen, und b) wie das basale Corticosteron und der Anstieg des Corticosterons auf einen akuten Stressor von der Situation des Nestlings abhängt (Alter, Schlupfreihenfolge und Körperkondition). Wir schauten auch, ob eine eventuelle Variation in der Kapazität des corticosteroidbindenden Globulins (CBG) die Konzentration des ungebundenen zirkulierenden Corticosterons verändert. Die Studie wurde bei freilebenden Turmfalken *Falco tinnunculus* durchgeführt, einer kleinen Greifvogelart, die vor allem Wühlmäuse jagt und Schlupfasynchronie zeigt.

In **Kapitel 1** untersuchten wir die Wirkung einer kürzlich entwickelten Methode zur Corticosteronverabreichung. Wir testeten, wie gut und regelmässig sich selbst abbauende Corticosteronpellets das zirkulierende Corticosteron bei Nestlingen von Turmfalken und Schleiereulen *Tyto alba* künstlich erhöhen. Gleichzeitig prüften wir, ob es Wechselwirkungen zwischen dem erhöhten Corticosteron und dem corticosteroidbindenden Globulin beziehungsweise der endogenen Corticosteronerhöhung auf das Handling gibt. Die Pellets induzierten während 2 bis 3 Tagen eine Erhöhung des zirkulierenden totalen Corticosterons. Dabei wurde anfangs ein Spitzenwert erreicht, der einem durch akuten Stress erhöhten Corticosteronspiegel entsprach. Bald jedoch sanken die Werte auf eine tiefere Konzentration. Während und bis mindestens 8 Tage nach der Implantierung löste das Handling keine akute Corticosteronerhöhung aus, was auf die Negativ-Feedback-Regulation des zirkulierenden Corticosterons hindeutet. 20 Tage nach der Implantierung war die Corticosteronantwort auf das Handling wieder wie vor der Implantierung. Während das totale Corticosteron künstlich erhöht war, war die Kapazität des Corticosteron-bindenden Globulins auch erhöht, was eine weniger starke Erhöhung des ungebundenen Corticosterons zur Folge hatte.

Die Wirkung einer solchen künstlichen Corticosteronerhöhung durch Pellets während der postnatalen Entwicklung auf verschiedene Wachstumsparameter und die Morphologie vor dem Ausfliegen bei Turmfalkennestlingen wurde in **Kapitel 2** untersucht. Während der 2 - 3 tägigen Erhöhung des Corticosterons in der Mitte der Nestlingszeit war - verglichen mit einer Placebo-Gruppe - das Flügelfedernwachstum signifikant auf 71 % reduziert, Hand- und Tarsuswachstum waren auf 14 % respektive 26 % reduziert und die Körpergewichtszunahme wurde gestoppt. Während der folgenden fünf Tage bis zum Tag 21 erreichte das Flügelfedernwachstum immer noch nur 84 % der Wachstumsrate der Placebogruppe, während Hand- und Tarsuswachstum und Körper-

gewichtszunahme wieder normal waren. Die Zunahme der Fettreserven mit dem Alter wurde durch die Corticosteronbehandlung nicht beeinflusst. Vor dem Ausfliegen war bei corticosteronbehandelten Nestlingen die Flügelfedernlänge 10 % kürzer, Hand und Tarsus 4 – 5 % kürzer und das Körpergewicht 9 % geringer als bei placebobehandelten Nestlingen. Feder-, Knochenwachstum und Körpergewichtszunahme waren also in verschiedenem Ausmass reduziert, was darauf hindeutet, dass die wachstumshemmende Wirkung von Corticosteron verschiedene Gewebe unterschiedlich stark betrifft. Das veranschaulicht, dass Corticosteron in die Kontrolle der Plastizität der Entwicklung involviert ist.

Eine vor einigen Jahren publizierte Hypothese schlägt vor, dass sich die adrenocorticale Stressantwort während der postnatalen Entwicklung bei Vögeln erst entwickeln soll, wenn der Nutzen von erhöhtem Corticosteron die schädlichen Effekte von Corticosteron auf Wachstum und Entwicklung aufwiegt. Im **Kapitel 3** untersuchten wir bei Turmfalkennestlingen drei Voraussagen, die aus dieser Hypothese entwickelt wurden. Erstens nahm die adrenocorticale Antwort auf das Handling und die menschliche Präsenz am Nest vom Tag 10 zum Tag 21 zu. Im gleichen Zeitraum entwickelten die Nestlinge ein Verteidigungsverhalten gegen Eindringlinge. Zweitens zeigten Nestlinge mit vielen Körperenergiereserven (geschätzt anhand der subkutanen Fettreserven in der Furcula) eine starke adrenocorticale Antwort auf einen akuten Stressor, während Nestlinge mit wenig Energiereserven eine reduzierte Antwort zeigten und damit zusätzliche Energieausgaben vermieden. Die basalen Corticosteronwerte waren negativ mit den Körperenergiereserven korreliert. Drittens zeigten sowohl die basalen Corticosteronwerte als auch die adrenocorticale Antwort auf das Handling keine Beziehung mit der Schlupfreihenfolge, sondern waren vorwiegend durch die Körperkondition bestimmt. Das Muster von abnehmenden Corticosteronkonzentrationen mit der Schlupfreihenfolge, das bei ad libitum gefütterten Nestlingen im Labor gefunden wurde, wird möglicherweise bei freilebenden Vögeln durch die Variation in der Körperkondition überlagert. Da das corticosteroidbindende Globulin nicht von einem der untersuchten Parameter abhängig war, zeigte das ungebundene Corticosteron ein ähnliches Muster wie das totale Corticosteron. Die Anpassung der adrenocorticalen Antwort während der postnatalen Entwicklung scheint adaptiv zu sein, um die negativen Wirkungen von erhöhtem Corticosteron auf den Phänotyp, welcher lebenslang beeinträchtigt werden kann, zu vermeiden.

In **Kapitel 4** untersuchten wir die Konsequenzen von Schlupfasynchronie aus der Perspektive des letztgeschlüpften Nestlings, statt wie üblich aus der Perspektive der Eltern. Die Brutreduktionshypothese erklärt das Auftreten von Schlupfasynchronie damit, dass die Brutgrösse durch Verhungern des letztgeschlüpften Nestlings bei Nahrungsknappheit adaptiv reduziert wird. Wir zeigen, dass die Strategie des letztgeschlüpften Nestlings mit der Nahrungsverfügbarkeit (jährliche Schwankungen im Beuteangebot und kurze Nahrungsverknappungen durch die Einschränkung des elterlichen Jagderfolgs durch Regen) variiert. Bei guten Bedingungen (kein Regen, viele Fettreserven in der Furcula) hatten die letztgeschlüpften Nestlinge tiefere basale Corticosteronwerte und eine höhere Flügelfedernwachstumsrate als die älteren Geschwister, was ein gewisses Aufho-

len im Wachstum erlaubt. Eine kurze Nahrungsverknappung (durch Regen) resultierte in höheren basalen Corticosteronwerten in den letztgeschlüpften Nestlingen verglichen mit den älteren Geschwistern. Die Fettreserven waren bei den letztgeschlüpften Nestlingen geringer als bei den älteren Geschwistern. Bei letztgeschlüpften Nestlingen mit hohen Corticosteronwerten waren das Flügelfedernwachstum und die Körpergewichtszunahme reduziert und die Sterblichkeit erhöht. Wir schliessen daraus, dass die letztgeschlüpften Nestlinge die Corticosteronkonzentration und Wachstumsstrategie konditionsabhängig anpassen, um das Beste aus ihrer schlechten Ausgangssituation zu machen.

Zusammengefasst zeigt diese Studie an einer Nesthocker-Art, dass das Stresshormon Corticosteron eine wichtige regulierende Rolle in der Reaktion auf variierende Bedingungen spielt und den Phänotyp beeinflusst. Die Erhöhung der Corticosteronkonzentration im Plasma als Reaktion auf einen akuten Stressor hingegen wird dem Alter und der Kondition angepasst, vermutlich um die adrenocorticale Antwort in Bezug auf die vorherrschenden externen und internen Bedingungen zu optimieren.

General introduction

General introduction

Unpredictable events and glucocorticoids

Free-living animals face predictable changes of their environment, e.g. seasonal changes in resource availability and climate, which they counteract with adjustments in morphology, physiology and behaviour. However, the physical and social environment comes up with a wide range of unpredictable challenges, which have the potential to threaten the homeostasis of the organism. Such unpredictable events, e.g. temperature extremes, a reduced access to trophic resources or social instability have the potential to cause physiological stress. Survival and reproductive success of animals (and thus their fitness) depend on both the adaptations to the overall conditions of their habitat and on efficient strategies of responding to unpredictable and unfavourable events (Sapolsky et al., 2000; summarized after Wingfield and Romero, 2001). In response to such unpredictable events (stressors), animals may change their physiology and behaviour and enter an emergency life-history stage (see Wingfield et al., 1998) to overcome the threatening situation.

In all vertebrates, the main physiological response to stressors consists of two components of the hypothalamo-pituitary-adrenal (HPA) axis (Norris, 1997). In a first wave, within seconds after the onset of a stressful event, e.g. a sudden predator attack, catecholamins are released from the sympathetic nervous system and put the animal in a state of combat (Vellucci, 1997; Sapolsky et al., 2000; Wingfield and Romero, 2001). When a stressor is slowly building up to chronic stress, e.g. prolonged poor food availability, this response is absent. Then, within minutes, a second wave follows with an increase in circulating levels of glucocorticoids (corticosterone in birds), through activation of the HPA axis (Buckingham et al., 1997a; Buckingham et al., 1997b; Sapolsky *et al.*, 2000). Elevated glucocorticoids have many behavioural and physiological effects: they suppress reproductive behaviour, regulate the immune system, increase gluconeogenesis and foraging behaviour, promote escape behaviour and night restfulness and promote recovery on return to normal life-history stage (summarized in Wingfield et al., 1998; Wingfield and Romero, 2001). This elevation of glucocorticoid levels helps the animal to cope with the dangerous situation by ensuring energy provision or provoking movement away from the threat. Thus glucocorticoids orchestrate, although not exclusively, the physiological, behavioural and developmental responses of an animal to stressful events.

After short perturbations, for example a predator attack or a short storm, normal activities are resumed shortly after. The ability, to react to such an acute stressor, can be assessed with the so called capture-stress-protocol (e.g. Wingfield and Romero, 2001), the capture and handling of a free-living animal is perceived as a predator attack and the following rise in glucocorticoids to this event can be measured by repeated blood sampling. Longer-term disturbances, examples include prolonged severe weather or a reduced food availability resulting in a negative energy balance of the individual, normally arise more slowly. Their effects eventually accumulate and normal activities

are disrupted. Elevated glucocorticoids then decrease after hours to days and thereafter the momentary life cycle stage is resumed or the next commenced.

While short-term elevations of glucocorticoids are adaptive by increasing the survival probability and allowing future reproductive success, long-term high elevations by chronic stressors have predominantly deleterious effects. Chronically high corticosterone elevations inhibit the reproductive system, suppress growth, promote severe protein loss, suppress the immune system and can result in severe neuronal cell death (summarized after Wingfield and Romero, 2001) and in free-living animals are only found close to death.

These stressor-induced high corticosterone levels are superimposed over the normal baseline (maintaining certain basic physiological functions) and possibly seasonally modulated intermediate corticosterone levels. These intermediate levels are directed by predictable environmental changes for the regulation of life-cycle stages and have regulatory and permissive functions (Sapolsky, 1992; Wingfield and Ramenofsky, 1999). Examples include dispersal (Silverin, 1997; Belthoff and Dufty, 1998; Dufty and Belthoff, 2001), preparation for migration in birds (Holberton et al., 1996; Landys-Ciannelli et al., 2002), or when leaving the nest in fledging altricial birds (Heath, 1997). It appears, that they can also be elicited by moderately stressful situations, such as suboptimal habitat quality (Marra and Holberton, 1998; Lanctot et al., 2003). The effects of these intermediate glucocorticoids levels are poorly known, though when persisting, they may have important permissive and regulatory physiological functions (Wingfield *et al.*, 1998; Sapolsky *et al.*, 2000).

The aim of any stress response is to avoid or overcome stress and to mitigate the negative effects of a persisting stressful situation. If moderate stress persists (e.g. poor food availability), intermediate levels of glucocorticoids adapt the physiology and behaviour so that fitness is still maximised under the prevailing conditions, although lower than under optimal conditions.

Downstream from the circulating corticosterone, plasma corticosteroid-binding-globulins (CBGs) can regulate the general availability of steroid to tissues, and direct the delivery of hormones to specific sites (Breuner and Orchinik, 2002). Free corticosterone, the unbound fraction of plasma corticosterone, is believed to be biologically active. CBG capacity and estimated free corticosterone therefore should be considered when examining the effects of corticosterone.

The adrenocortical response to stress during postnatal development

During growth and development, the phenotype of an individual is shaped by maternal and paternal effects and the environment (reviewed in Lindström, 1999). The persistence and strength of the effects of adverse conditions during growth and development depend on the possibilities to catch-up later, while compensatory growth is also associated with costs (reviewed in Metcalfe and Monaghan, 2001). Glucocorticoids presumably play a crucial role during growth and development in view of unpredictable environmental perturbations. Generally, hormones can interact with early development and have life-long effects (Dufty et al., 2002). In critical situations, glucocorticoids

mobilize energy for maintenance and thus increase short-term survival by catabolizing protein stores and suppressing anabolic processes like growth (reviewed in Schreck, 1993). Behavioural effects of corticosterone may result in the maximisation of nutritional input. While precocial chicks, similar as adults, can enhance food searching behaviour (Astheimer et al., 1992), nest-bound, altricial nestlings are only able to improve food intake indirectly, by increasing begging or aggression against siblings (Kitaysky et al., 2001b; Kitaysky et al., 2003; Loiseau et al., 2008).

In most altricial nestlings investigated until now, the adrenocortical response to handling has been shown to develop during postnatal development (e.g. Sims and Holberton, 2000). It is believed that any unnecessary suppression of growth by corticosterone during early development is avoided. The rise in corticosterone to an acute stressor develops in parallel to the ability of the nestling to react to these stressors. However, potential functions of corticosterone during postnatal development, e.g. in metabolism (e.g. body condition) and behaviour (e.g. anti-predator-behaviour) are largely unexplored. In asynchronous hatching species, the relationship between corticosterone and brood hierarchy is unclear. Different studies found contradicting results (Sockman and Schwabl, 2001; Love et al., 2003).

Different environmental stressors like food-restriction caused by a dietary restriction (Kitaysky et al., 1999; Kitaysky et al., 2001a) or suboptimal habitat quality (Suorsa et al., 2003; Blas et al., 2005) have been found to elevate baseline corticosterone of altricial nestling to intermediate levels, similarly as social subordination (Nunez-de la Mora et al., 1996) and human presence at nest (Fridinger et al., 2007).

Short- and long-term effects of elevated glucocorticoids during post-natal development

The available studies suggest that elevated corticosterone levels during the nestling period may have long-lasting and profound effects on many aspects of the future life, i.e. they may profoundly shape the phenotype. Under lab conditions, elevated corticosterone levels have been found to suppress growth in precocial species (e.g. Hull et al., 2007; Dong et al., 2007). However, only a few studies investigated the effect of corticosterone on growth in altricial nestlings in the lab (Spencer et al., 2003; Wada and Breuner, 2008), while such studies in free-living altricial nestlings are missing. Food restriction studies found a hierarchy in structural growth allocation, favouring presumably the most sensitive tissues (e.g. Moe et al., 2004; Benowitz-Fredericks et al., 2006). However, it is not known, whether corticosterone is involved in growth allocation by having different effects on different tissues. While compensatory growth after nutritional restriction has been studied extensively (reviewed in Metcalfe and Monaghan, 2001), potential compensatory growth after an elevation in corticosterone levels has not been investigated so far. Further, T-cell mediated immunity has been shown to negatively correlate with corticosterone levels in altricial nestlings (Saino et al., 2003). Long-term effects of elevated corticosterone during postnatal development (in

altricial nestlings) on morphology are not investigated in detail, but long-term interferences have been found on song performance (Spencer *et al.*, 2003) and cognition (Kitaysky *et al.*, 2003).

The Eurasian kestrel *Falco tinnunculus*

The Eurasian kestrel *Falco tinnunculus* is a small raptor species inhabiting open landscapes all over Europe except Iceland and above 70° N in Siberia EBCC (Hagemeijer and Blair, 1997) and hunting on small vertebrates and large insects. Similarly as in Northern Europe (Korpimäki and Norrdahl, 1991) and contrary to Mediterranean Europe (Costantini et al., 2005), voles are the preferred prey in middle Europe. In Switzerland, the common vole *Microtus arvalis* is presumably the most abundant vole species in agricultural habitats (Aschwanden et al., 2007), and the main prey of kestrels (own observation). As in Northern Europe (Korpimäki et al., 2004), but to a lesser extent, voles show population cycles in Switzerland (e.g. described for the water vole Arvicola terrestris, Saucy, 1994), resulting in a yearly fluctuating prey availability and a corresponding fluctuation of kestrel breeding pairs and reproductive success (own observation).

Kestrels show reversed size dimorphism, females being about 5% larger and 10 - 15% heavier than males (Glutz von Blotzheim, 1993). Between the beginning of April and the end of May, the female lays 3 to 7 eggs (own unpublished data) and incubates them for about 29 days (Piechocki, 1982), usually beginning after laying the third egg. This results in partial hatching asynchrony and often in brood reduction (Lack, 1954; Wiebe et al., 1998; Wiehn et al., 2000). The female broods the nestlings until about day 10. She distributes the food brought by the male to the chicks by tearing off small pieces and presenting them carefully to the young next to her. Contrary to other representatives of the Falconiformes, nestling falcons neither show aggression between siblings nor siblicide (Newton, 1979). They just push themselves to the best place to receive food from the female or fight for prey items when they get older. The male first provisions the laying and incubating female and then the whole family. With about 15 days, nestlings are able to process the delivered prey by themselves, while the female also often hunts to supply the nestlings with food (Village, 1990). 32 – 39 days after hatching, nestlings fledge and stay for another 13 – 26 days within the family (own unpublished data).

The study area in North-western Switzerland

The field work was performed in the Jura mountains and river valleys of the cantons Baselland and Aargau in North-western Switzerland (7°60'E / 47°30'N), where Eurasian kestrels breed in nest boxes mounted on agricultural buildings in open rural landscapes. Six subplots supplied with 150 nest boxes within an area covering approximately 600 km^2 were investigated. These subplots (Baselland: *Therwil*, *Unteres Baselbiet*, *Oberes Baselbiet*; Aargau: *Fricktal*, *Bözberg*, *Surbtal*) vary

in kestrel densities, mean clutch size and prevailing land use and are situated between 275 and 710 m a.s.l.

Research goals

The main objectives of this thesis were twofold:

1) In an experimental approach, the effects of elevated glucocorticoids on structural growth should be investigated in free-living, altricial nestlings during post-natal development. Looking at potential long-term effects on morphology, I wanted to examine the role of corticosterone in shaping the phenotype during and after an environmental perturbation, mimicked by artificially elevated corticosterone.

2) To reveal the relevance of elevated corticosterone levels in the natural context, I wanted to explore the natural variation in baseline and handling-induced corticosterone in free-living, altricial nestlings in an observational study. We aimed at explaining the natural variation in corticosterone levels by ontogenesis (development of the adrenocortical response), the physical (food availability) and social (brood hierarchy) environment and physiology (condition).

Outline of the thesis

Chapter 1 Effects of corticosterone pellets on baseline and stress-induced corticosterone and corticosteroid-binding-globulin

In this first chapter, we evaluated the performance of self-degradable corticosterone pellets to artificially elevate circulating corticosterone levels in free-living kestrel and barn owl *Tyto alba* nestlings. We were interested in the plasma levels and the time course of corticosterone resulting from the pellets and investigated potential interactions with the adrenocortical response to handling and corticosteroid-binding-globulin.

Chapter 2 Effects of a short period of elevated circulating corticosterone on postnatal growth in free-living Eurasian kestrels *Falco tinnunculus*

We then investigated the effects of this experimental corticosterone elevation of 2 – 3 days by the pellets in the middle of the nestling stage on growth. Without nutritional restriction, the impact of elevated corticosterone on feather (primary 8) and bone (hand, tarsus) growth, body mass increase and the development of furcular fat score were monitored before, during and after the artificial elevation in corticosterone, allowing us to record/include any compensatory growth before fledging.

Chapter 3 Development of the adrenocortical response to stress in Eurasian kestrel nestlings *Falco tinnunculus*: the importance of condition and brood hierarchy

In this chapter, we explored the natural variation of baseline and handling-induced corticosterone levels in the first and second half of the nestling stage in free-living kestrel nestlings. We investigated, whether the behavioural reaction to human presence at the nest in the second half of the nestling stage is paralleled by an increase in corticosterone, sustaining this response. We further unravelled potential correlates of corticosterone levels with age (developmental stage), condition (measured as furcular fat score) and brood hierarchy.

Chapter 4 Strategies of the last-hatched nestling under varying food availability in asynchronous broods of the Eurasian kestrel: corticosterone, condition and growth

This chapter is dedicated to the last-hatched nestling of kestrel broods which hatch asynchronously. When food is restricted, often the last hatched nestlings dies. From the point of view of the last-hatched nestling, we unravel the dynamics of corticosterone and growth in a varying environment (long-term food shortage by fluctuating prey and short-term impairments by rain). We investigated whether the last-hatched nestling adopts a different strategy than its older siblings under good environmental conditions and under restricted food supply.

References

Aschwanden, J., Holzgang, O., Jenni, L., 2007. Importance of ecological compensation areas for small mammals in intensively farmed areas. Wildlife Biology 13, 150-158.

Astheimer, L.B., Buttemer, W.A., Wingfield, J.C., 1992. Interactions of corticosterone with feeding, activity and metabolism in passerine birds. Ornis Scandinavica 23, 355-365.

Belthoff, J.R., Dufty, A.M., 1998. Corticosterone, body condition and locomotor activity: A model for dispersal in screech-owls. Animal Behaviour 55, 405-415.

Benowitz-Fredericks, Z.M., Kitaysky, A.S., Thompson, C.W., 2006. Growth and allocation in captive common murre (*Uria aalge*) chicks. The Auk 123, 722-734.

Blas, J., Baos, R., Bortolotti, G.R., Marchant, T., Hiraldo, F., 2005. A multi-tier approach to identifying environmental stress in altricial nestling birds. Functional Ecology 19, 315-322.

Breuner, C.W., Orchinik, M., 2002. Plasma binding proteins as mediators of corticosteroid action in vertebrates. Journal of Endocrinology 175, 99-112.

Buckingham, J.C., Cowell, A.-M., Gillies, G.E., Herbison, A.E., Steel, J.H., 1997a. The neuroendocrine system: anatomy, physiology and responses to stress. In: Buckingham, J.C., Gillies, G.E., Cowell, A.M. (Eds.), Stress, stress hormones and the immune system. John Wiley & Sons, Chichester, pp. 9-47.

Buckingham, J.C., Gillies, G.E., Cowell, A.-M., 1997b. Stress, stress hormones and the immune system. John Wiley & Sons, Chichester.

Costantini, D., Casagrande, S., Di Lieto, G., Fanfani, A., Dell'Omo, G., 2005. Consistent differences in feeding habits between neighbouring breeding kestrels. Behaviour 142, 1403-1415.

Dong, H., Lin, H., Jiao, H.C., Song, Z.G., Zhao, J.P., Jiang, K.J., 2007. Altered development and protein metabolism in skeletal muscles of broiler chickens (*Gallus gallus domesticus*) by corticosterone. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 147, 189-195.

Dufty, A.M., Belthoff, J.R., 2001. Proximate mechanisms of natal dispersal: the role of body condition and hormones. In: Clobert, J., Danchin, E., Dhondt, A., Nichols, J.D. (Eds.), Dispersal. Oxford University Press, Oxford, pp. 217-229.

Dufty, A.M., Clobert, J., Moller, A.P., 2002. Hormones, developmental plasticity and adaptation. Trends in Ecology and Evolution 17, 190-196.

Fridinger, R.W., O'Reilly, K.M., an Kildaw, S., Loren Buck, C., 2007. Removal of a nest-mate elicits an age-dependent increase in plasma corticosterone of nestling Black-legged Kittiwakes. Journal of Field Ornithology 78, 93-99.

Glutz von Blotzheim, U.N., 1993. Handbuch der Vögel Mitteleuropas. Aula Verlag, Wiesbaden.

Hagemeijer, W.J.M., Blair, M.J., 1997. The EBCC Atlas of European Breeding Birds: Their Distribution and Abundance. T. & A. D. Poyser London.

Heath, J., 1997. Corticosterone levels during nest departure of juvenile American kestrels. Condor 99, 806-811.

Holberton, R., Parrish, J.D., Wingfield, J.C., 1996. Modulation of the adrenocortical stress response in neotropical migrants during autumn migration. Auk 113, 558-564.

Hull, K.L., Cockrem, J.F., Bridges, J.P., Candy, E.J., Davidson, C.M., 2007. Effects of corticosterone treatment on growth, development, and the corticosterone response to handling in young Japanese quail (*Coturnix coturnix japonica*). Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 148, 531-543.

Kitaysky, A.S., Kitaiskaia, E.V., Piatt, J.F., Wingfield, J.C., 2003. Benefits and costs of increased levels of corticosterone in seabird chicks. Hormones and Behavior 43, 140-149.

Kitaysky, A.S., Kitaiskaia, E.V., Wingfield, J.C., Piatt, J.F., 2001a. Dietary restriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. Journal of Comparative Physiology – B: Biochemical, Systemic, and Environmental Physiology 171, 701-709.

Kitaysky, A.S., Piatt, J.F., Wingfield, J.C., Romano, M., 1999. The adrenocortical stress-response of Black-legged Kittiwake chicks in relation to dietary restrictions. Journal of Comparative Physiology – B: Biochemical, Systemic, and Environmental Physiology 169, 303-310.

Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 2001b. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. Behavioral Ecology 12, 619-625.

Korpimäki, E., Brown, P.R., Jacob, J., Pech, R.P., 2004. The Puzzles of Population Cycles and Outbreaks of Small Mammals Solved? Bioscience 54, 1071-1079.

Korpimäki, E., Norrdahl, K., 1991. Numerical and functional responses of kestrels, short-eared owls and long-eared owls to vole densities. Ecology 72, 814-826.

Lack, D., 1954. The natural regulation of animal numbers. Clarendon Press, Oxford.

Lanctot, R.B., Hatch, S.A., Gill, V.A., Eens, M., 2003. Are corticosterone levels a good indicator of food availability and reproductive performance in a kittiwake colony? Hormones and Behavior 43, 489-502.

Landys-Ciannelli, M.M., Ramenofsky, M., Piersma, T., Jukema, J., Castricum, R.G., Wingfield, J.C., 2002. Baseline and stress-induced plasma corticosterone during long-distance migration in the bar-tailed godwit, *Limosa lapponica*. Physiological and Biochemical Zoology 75, 101-110.

Lindström, J., 1999. Early development and fitness in birds and mammals. Trends in Ecology and Evolution 14, 343-348.

Loiseau, C., Sorci, G., Dano, S., Chastel, O., 2008. Effects of experimental increase of corticosterone levels on begging behavior, immunity and parental provisioning rate in house sparrows. General and Comparative Endocrinology 155, 101-108.

Love, O.P., Bird, D.M., Shutt, L.J., 2003. Plasma corticosterone in American kestrel siblings: Effects of age, hatching order, and hatching asynchrony. Hormones and Behavior 43, 480-488.

Marra, P.P., Holberton, R.L., 1998. Corticosterone levels as indicators of habitat quality: Effects of habitat segregation in a migratory bird during the non-breeding season. Oecologia 116, 284-292.

Metcalfe, N.B., Monaghan, P., 2001. Compensation for a bad start: grow now, pay later? Trends in Ecology and Evolution 16, 254-260.

Moe, B., Brunvoll, S., Mork, D., Brobakk, T.E., Bech, C., 2004. Developmental plasticity of physiology and morphology in diet-restricted European shag nestlings (*Phalacrocorax aristotelis*). Journal of Experimental Biology 207, 4067-4076.

Newton, I., 1979. Population Ecology of Raptors. T & A D Poyser, Berkhamsted.

Norris, D.O., 1997. Vertebrate endocrinology.

Nunez-de la Mora, A., Drummond, H., Wingfield, J.C., 1996. Hormonal correlates of dominance and starvation-induced aggression in chicks of the blue-footed booby. Ethology 102, 748-761.

Piechocki, R., 1982. Der Turmfalke *Falco tinnunculus*: seine Biologie und Bedeutung für die biologische Schädlingsbekämpfung. Die neue Brehm-Bücherei, Wittenberg Lutherstadt.

Saino, N., Suffritti, C., Martinelli, R., Rubolini, D., Moller, A.P., 2003. Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (*Hirundo rustica*). Behavioral Ecology 14, 318-325.

Sapolsky, R.M., 1992. Stress, the aging brain, and the mechanism of neuron death. MIT Press, Cambridge, Massachusetts.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21, 55-89.

Saucy, F., 1994. Density dependence in time series of the fossorial form of the water vole, *Arvicola terrestris*. Oikos 71, 381-392.

Schreck, C.B., 1993. Glucocorticoids: Metabolism, Growth, and Development. In: Schreibman, M.P., Scanes, C.G., Pang, P.K.T. (Eds.), The endocrinology of growth, development, and metabolism in vertebrates. Academic Press, San Diego, pp. 367-392.

Silverin, B., 1997. The stress response and autumn dispersal behaviour in willow tits. Animal Behaviour 53, 451-459.

Sims, C.G., Holberton, R.L., 2000. Development of the corticosterone stress response in young Northern Mockingbirds (*Mimus polyglottos*). General and Comparative Endocrinology 119, 193-201.

Sockman, K.W., Schwabl, H., 2001. Plasma corticosterone in nestling American kestrels: Effects of age, handling stress, yolk androgens, and body condition. General and Comparative Endocrinology 122, 205-212.

Spencer, K.A., Buchanan, K.L., Goldsmith, A.R., Catchpole, C.K., 2003. Song as an honest signal of developmental stress in the zebra finch (*Taeniopygia guttata*). Hormones and Behavior 44, 132-139.

Suorsa, P., Huhta, E., Nikula, A., Nikinmaa, M., Jäntti, A., Helle, H., Hakkarainen, H., 2003. Forest management is associated with physiological stress in an old-growth forest passerine. Proceedings of the Royal Society of London – Series B: Biological Sciences 270, 963-969.

Vellucci, S.V., 1997. The autonomic and behavioural responses to stress. In: Buckingham, J.C., Gillies, G.E., Cowell, A.-M. (Eds.), Stress, stress hormones and the immune system. John Wiley & Sons, Chichester, pp. 49-70.

Village, A., 1990. The Kestrel. T & A D Poyser, London.

Wada, H., Breuner, C.W., 2008. Transient elevation of corticosterone alters begging behavior and growth of white-crowned sparrow nestlings. Journal of Experimental Biology 211, 1696-1703.

Wiebe, K.L., Korpimäki, E., Wiehn, J., 1998. Hatching asynchrony in Eurasian kestrels in relation to the abundance and predictability of cyclic prey. Journal of Animal Ecology 67, 908-917.

Wiehn, J., Ilmonen, P., Korpimäki, E., Pahkala, M., Wiebe, K.L., 2000. Hatching asynchrony in the Eurasian kestrel Falco tinnunculus: An experimental test of the brood reduction hypothesis. Journal of Animal Ecology 69, 85-95.

Wingfield, J.C., Maney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M., Richardson, R.D., 1998. Ecological Bases of Hormone-Behavior Interactions: The "Emergency Life History Stage". American Zoologist 38, 191-206.

Wingfield, J.C., Ramenofsky, M., 1999. Hormones and the behavioural ecology of stress. In: Balm, P.H.M. (Ed.), Stress physiology in animals. CRC Press, Sheffield, pp. 1-51.

Wingfield, J.C., Romero, L.M., 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates. In: McEwen, B., Goodman, H.M. (Eds.), Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms Oxford University Press, New York, pp. 211-234.

Chapter 1

Effects of corticosterone pellets on baseline and stress-induced corticosterone and corticosteroid-binding-globulin

Claudia Müller, Bettina Almasi, Alexandre Roulin, Creagh W. Breuner, Susanne Jenni-Eiermann and Lukas Jenni

General and Comparative Endocrinology (2009) 160: 59 - 66

Abstract

Exogenous administration of glucocorticoids is a widely used and efficient tool to investigate the effects of elevated concentrations of these hormones in field studies. Because the effects of corticosterone are dose and duration dependent, the exact course of plasma corticosterone levels after exogenous administration needs to be known. We tested the performance of self-degradable corticosterone pellets (implanted under the skin) in elevating plasma corticosterone levels. We monitored baseline (sampled within 3 min after capture) total corticosterone levels and investigated potential interactions with corticosteroid-binding-globulin (CBG) capacity and the endogenous corticosterone response to handling in Eurasian kestrel *Falco tinnunculus* and barn owl *Tyto alba* nestlings. Corticosterone pellets designed for a 7-day-release in rodents elevated circulating baseline total corticosterone during only 2 - 3 days compared to placebo-nestlings. Highest levels occurred 1 - 2 d after implantation and levels decreased strongly thereafter. CBG capacity was also increased, resulting in a smaller, but still significant, increase in baseline free corticosterone levels. The release of endogenous corticosterone as a response to handling was strong in placebo-nestlings, but absent 2 and 8 days after corticosterone pellet implantation. This indicates a potential shutdown of the hypothalamo-pituitary-adrenal axis after the 2 to 3 days of elevated baseline corticosterone levels. 20 days after pellet implantation, the endogenous corticosterone response to handling of nestlings implanted with corticosterone pellets attained similar levels as in placebo-nestlings. Self-degradable pellets proved to be an efficient tool to artificially elevate circulating baseline corticosterone especially in field studies, requiring only one intervention. The resulting peak-like elevation of circulating corticosterone, the concomitant elevation of CBG capacity, and the absence of an endogenous corticosterone response to an acute stressor have to be taken into account.

1. Introduction

Exogenous administration of glucocorticoids is a widely used and efficient tool to investigate the effects of elevated concentrations of these hormones. Methods to artificially elevate glucocorticoids include single or repeated injections (e.g. Remage-Healey and Romero, 2002; Loiseau et al., 2008), adding hormone to the food or drinking water (e.g. Breuner et al., 1998; Hiebert et al., 2000; Hull et al., 2007), or implantation of a silastic tube filled with crystalline hormone (e.g. Silverin, 1986; Wingfield and Silverin, 1986; Kitaysky et al., 2001), a gelling material containing hormone (French et al., 2007), a mini-infusion pump (Donker and Beuving, 1989) or an osmotic pump (Horton et al., 2007). These various forms of administration have different advantages and disadvantages. Injections imply repeated handling with concurrent internal adrenocortical responses when hormone levels should be elevated over days. While the intake of glucocorticoids with food or water is not invasive, the amount of assimilated glucocorticoids depends on the quantity of food or water ingested and can vary between individuals. Additionally, repeated injections and hormone consumption are very difficult to apply in free-living animals. Glucocorticoid-releasing implants or osmotic pumps require only one intervention followed by the release of a given amount of glucocorticoids over a longer period of time and are often the method of choice in field studies. However, removal of these implants requires a second intervention. An alternative method is the implantation of self-degradable corticosterone releasing pellets, which should provide a constant release by biodegradation of the matrix and do not require subsequent removal. This method has rarely been used in experiments with wild bird species until now (e.g. Pravosudov, 2003; Bourgeon and Raclot, 2006; Bonier et al., 2007).

In most studies the effect of corticosterone administration on circulating corticosterone is not well documented, although the levels of circulating corticosterone attained and the duration of the elevation are supposed to be decisive, since the effects of corticosterone are dose dependent (e.g. Romero, 2004) and the duration of the exposure to elevated levels is crucial. The time course of circulating baseline (sampled within 3 min after capture) corticosterone after administering corticosterone in birds has been followed in only a few studies by measuring circulating total corticosterone at a few specific time points, e.g. after several days (e.g. Kitaysky et al., 2001). The interactions further up- and downstream from corticosterone during and after an exogenous corticosterone administration have been investigated predominantly in mammals (e.g. Feldman et al., 1979; Zhao et al., 1997) and only rarely in birds. Only one study investigated the effect of corticosterone administration on corticosteroid-binding-globulin (CBG) (Breuner et al., 2003b). We know of no study examining the response to an acute stressor after the period when circulating corticosterone was elevated

The aim of this paper is to report our experiences with self-degradable corticosterone-releasing pellets in an extended field study with nestling Eurasian kestrels *Falco tinnunculus* and barn owls *Tyto alba*. Specifically, we investigated (a) the time course of total circulating corticosterone during 20 days after implantation, (b) whether CBG capacity and estimated free corticosterone

levels were affected by corticosterone administration, and (c) whether the response of circulating corticosterone levels to an acute stressor (handling) was affected by corticosterone administration. We predicted that CBG capacity would increase with elevated corticosterone levels and that the negative feedback regulation would decrease the endogenous response to an acute stressor in cort-nestlings with elevated corticosterone compared to placebo-nestlings. For aim (b) and (c) we were interested in the acute effects of the implant and in potential mid-term effects after circulating baseline corticosterone had returned to normal levels.

2. Material and Methods

2.1. Study species and study sites

The Eurasian Kestrel is a small, diurnal raptor. Usually, the female starts incubation after laying the third of 4 - 6 eggs, hence, the three oldest nestlings have about the same age. The nestlings stay in the nest for 32 to 39 days and reach their maximal body mass around day 23. Fieldwork was performed in North-western Switzerland (47°25'N / 7°50'E), where kestrels raise their young in nest boxes mounted on agricultural buildings in open rural landscapes. Mean brood size (± SD) of the investigated broods at the day of implantation was 4.4 ± 0.96 nestlings, mean hatching date was on 7 June ± 13 days.

The barn owl is a medium-sized, nocturnal owl species producing clutches of 2 - 11 eggs. Incubation starts after laying the first egg and only females incubate. The laying intervals of two to three days entail a pronounced within-brood age hierarchy. The nestlings reach their maximum body mass with 40 days and fledge with about 56 days of age. Barn owls were investigated in Western Switzerland (46°49'N / 06°56'E), where they breed in nest boxes attached on barns and farm buildings. Mean brood size (± SD) of the investigated broods at the day of implantation was 5.8 ± 1.54 nestlings, mean hatching date was on 3 June ± 33 days.

2.2. Experimental corticosterone treatment

The experiment was carried out in 109 kestrel nestlings of 30 broods (13 in 2004 and 17 in 2005) and 208 barn owl nestlings of 73 broods (33 in 2004, 19 in 2005, 21 in 2006). Hatching date was determined through regular nest box controls.

Two randomly selected nestlings out of the four oldest within a brood were implanted with a self-degradable corticosterone-releasing pellet on nestling day 13 (mean age ± SD: 13.2 ± 1.4 days) in the kestrel and nestling day 25 (mean age 27 ± 5 days) in barn owl nestlings. Pellets designed for a 7-day-release in rodents were obtained from *Innovative Research of America* (Sarasota, Florida, U.S.). Because kestrels were lighter in body mass (mean ± SD: 165 ± 22 g at implantation) than barn owls (294 ± 69 g at implantation), we implanted a 10 mg corticosterone pellet (cat

C-111) in nestling kestrels and a 15 mg corticosterone pellet (cat # G-111) in nestling barn owls. The other two of the four oldest siblings were implanted with a corresponding placebo pellet, creating two treatment groups (cort- and placebo-nestlings). Before implantation, body mass (kestrels: t = 1.44, df = 107, p = 0.153; barn owls: t = 0.71, df = 206, p = 0.478), nestling age (kestrels: t = 0.56, df = 107, p = 0.577; barn owls: t = 0.28, df = 206, p = 0.783) and the proportion of the sexes (kestrels: X^2 = 0.01, df = 1, p = 0.922; barn owls: X^2 = 0.09, df = 1, p = 0.752) did not differ between cort- and placebo-nestlings. The pellets were placed under the skin of the flank above the knee through a small incision. The pellets are very sensitive to alcoholic solvents, even when seemingly evaporated (based on our own observations in previous trials, and personal communication by the provider). Therefore, to prevent accelerated corticosterone release, the skin was not disinfected. The incision was closed with tissue adhesive (Histoacryl®, Braun, Germany). If more than four siblings were present, the fifth and following were not implanted. All methods described in this study were approved by the Swiss committee for animal research (animal experiment permit n°274 from the Cantonal Veterinarian Office of Baselland for kestrels and n° 1736 from the Veterinarian Office of Vaud for barn owls).

Pellet implantation and corticosterone treatment did not affect survival of nestlings. In kestrels nestlings, between nestling day 13 and fledging, 54 of 56 placebo-nestlings (96.4 %) and 60 of 61 cort-nestlings (98.4 %) survived, while 242 of 260 nestlings not treated with a pellet survived (93.1 %; nestlings of other broods at our study site during the study years which were only measured and blood sampled). In barn owls, 40 of 43 cort-nestlings (93.0 %) and 42 of 43 placebo-nestlings (97.7 %) survived until fledging, while 159 of 179 non-treated nestlings (88.8 %) survived until fledging. All differences in survival between cort-implanted, placebo-implanted and non-implanted birds were not significant (kestrel: Pearson X^2 = 3.12, d.f. = 2, p = 0.210; barn owl: Pearson X^2 = 3.57, d.f. = 2, p = 0.168).

2.3. Blood sampling

We took baseline blood samples in kestrel nestlings 3 days before implantation, on the day of implantation (day 0) and 3 and 8 days after implantation. In a subgroup of kestrel nestlings we took an additional baseline sample 1 or 2 days after implantation. All baseline samples were taken within 3 min after taking nestlings out of the nest box in batches of two. There was no effect of batch number or the time between first arrival and bleeding on baseline corticosterone (F = 0.57, d.f. = 2, p = 0.567). In barn owls we collected a blood sample within 3 minutes after having opened the nest box on the day of implantation and 6 and 20 days after implantation (blood samples taken after 3 min were not used for this study); a subgroup was also sampled 2 or 3 days after implantation. In addition, we had baseline samples of 5 cort-nestlings of 4 broods 1 day after implantation which we used to demonstrate whether corticosterone levels were not pharmacologically high, but we did not include them in the statistical tests, because the corresponding placebo samples were

missing. After taking the first blood sample, the nestlings were measured, weighed and then held in a cloth bag. The adrenocortical response to handling was assessed by taking a second blood sample about 17 min (mean ± SD: 16.9 ± 1.4 min, range: 15 - 22) after taking the nestlings out of the nest box in kestrels and 19 minutes (19.13. ± 2.92 min, range: 15 - 25) in barn owls. Handling-induced corticosterone levels did not vary within these time ranges (Placebo-nestlings: $p > 0.242$). Blood was sampled by puncturing the alar vein and collected in heparinized capillary tubes. Within 30 minutes, the blood was centrifuged in Eppendorf tubes and the plasma immediately stored in liquid nitrogen in the field and at –20°C once in the laboratory.

2.4. Corticosterone assay

Plasma corticosterone concentration was determined using an enzyme immuno assay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988). 5 µl plasma was added to 195 µl water, and from this solution we extracted corticosterone with 4 ml dichloromethane, which was re-dissolved in phosphate buffer and measured in triplicates in the enzyme-immunoassay. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, Progesterone 0.004%, 18-OH-DOC 0.01%, Cortisol 0.12%, 18-OH-B 0.02% and Aldosterone 0.06%) was 1:8'000. HRP (1:400'000) linked to corticosterone served as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. Plasma pools from chickens with two different corticosterone concentrations were included as internal controls on each plate. In 38 cases the concentration was below the detection threshold, and in this case the determination was repeated with 10 µl plasma. If the concentration was still below the detection threshold (27 samples), the value of the lowest detectable concentration (1 ng ml^{-1}) was assigned. Intra-assay variation ranged from 4.5 to 13.4 % and inter-assay variation from 9.6 to 23.0 %, depending on the concentration of the internal control and the year of determination.

2.5. Corticosteroid-binding-globulin

The affinity and capacity of corticosteroid-binding-globulin (CBG) was measured with a radioligand-binding assay with tritiated corticosterone following Breuner et al. (2003a). For point sample analysis, plasma (10-15 µl in the kestrel, 5 µl in the barn owl) was stripped of endogenous steroids with 2 parts of dextran-coated charcoal (0.1% dextran, 1% Norit A charcoal in 50 mM Tris) for 30 minutes at room temperature. Outside this stripping procedure, the plasma was maintained below 4°C. The final assay dilution of kestrel plasma samples was 1:99, those of the barn owl samples 1:450. The binding assay was carried out in 50 nM Tris buffer at 4°C and terminated after 2 hours. 1 hour before filtering, glass fiber filters (Whatman) were soaked in 25 nM Tris with 0.3% polyethylenimine. After filtration, filters were rapidly rinsed with 3 rinses of 3 ml ice-cold 25 nM Tris. Point

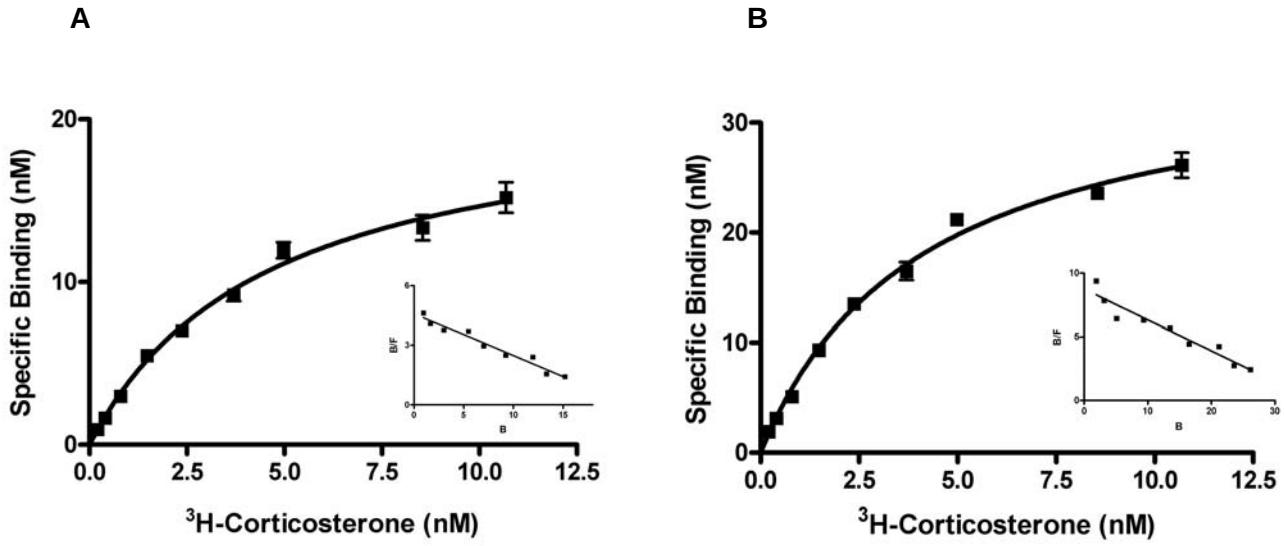

Fig. 1. Equilibrium saturation binding curve demonstrating specific binding of ^{3}H-corticosterone to A) Eurasian kestrel and B) barn owl plasma as a function of increasing concentrations of radiolabeled corticosterone. Points indicate means ± SE. The inlay is the Scatchard-Rosenthal replot of the data.

sample analysis was performed with individual plasma samples, for the saturation analyses pooled samples were run. For the saturation analyses, 0.25-12 nM [^{3}H]Corticosterone were incubated with pooled plasma of each species with and without 1µM unlabeled corticosterone. 20 nM [^{3}H]Corticosterone was employed to estimate CBG capacity in individual birds. Affinity estimates obtained from equilibrium saturation analysis (Kd = 4.59 and 4.11 nM in kestrels and barn owls, respectively, Figures 1a and b) indicated that this ligand concentration occupies ~80 % of total binding sites. For analysis, the samples were corrected to 100 % capacity within each assay. The intra-assay variation was 5.7 and 7.1 %, the inter-assay variation 7.58 and 17.1% (kestrel and barn owl samples determined separately).

The equation of Barsano and Baumann (1989) was used to estimate free corticosterone titers from total corticosterone concentrations and CBG binding parameters

$$H_{free} = 0.5 \times \left[H_{total} - B_{max} - 1/K_a\right] \pm \sqrt{(B_{max} - H_{total} + 1/K_a)^2 + 4(H_{total}/K_a)}$$

where H_{free} is free Hormone, H_{total} is total Hormone, B_{max} is total binding capacity of CBG, and K_a = 1/dissociation constant (K_d) (all values in nM). Corticosterone was analyzed in all baseline and handling induced blood samples, CBG capacity was measured in all baseline kestrel and barn owl samples and a subsample of the handling induced kestrel samples (a subsample of day 10, all samples of day 21).

2.6. Statistical analyses

To analyse the overall effect of corticosterone treatment on circulating corticosterone we performed mixed-models (REML, Genstat 10) separately with total corticosterone, CBG capacity and free

corticosterone as dependent variables. We included treatment (corticosterone *versus* placebo) and time after implantation (number of days after implantation) and their interaction as fixed factors and nestling identity nested in broods as random factors to correct for the dependence of siblings within a brood.

The effect of corticosterone treatment on the response to handling was analyzed separately for total and free corticosterone and CBG capacity by running a repeated measures analysis including baseline and stress-induced levels of those days with two blood samples per individual. The model included time after implantation, treatment, blood sample (baseline *versus* handling induced) and their interactions as fixed factors, and nestling identity nested in brood as random factors. To compare corticosterone levels of baseline and handling-induced blood samples and CBG capacity between treatment groups at the different sampling days, we performed post-hoc tests for each day separately. Treatment was included as fixed and brood as random factor in these mixed models and significance levels were adjusted according to Bonferroni (Sokal and Rohlf, 2000). Kestrel and barn owl data were analysed in separate models.

3. Results

3.1. Effect of corticosterone pellets on baseline total and free corticosterone levels and CBG capacity

Implanting a corticosterone pellet had a highly significant effect on the plasma concentration of total corticosterone in both species (Table 1, 2). Before implantation, there was no difference in total corticosterone level between cort- and placebo-nestlings in both species (Fig. 2A, 2B). Total corticosterone in kestrel cort-nestlings was significantly elevated over placebo-nestlings 1 day after

Table 1. Effect of corticosterone pellets on plasma levels of total baseline corticosterone, CBG capacity and estimated free corticosterone in kestrel nestlings. *Treatment group* refers to 56 nestlings implanted with a corticosterone pellet and 53 nestlings implanted with a placebo pellet. *Time after implantation* refers to blood samples taken 3 and 0 days before and 3 and 8 days after implantation from all nestlings and 1 or 2 days after implantation from 25 nestlings (470 measurements in total). The results from a mixed model analysis are given with nestling identity nested in broods as random factors.

	Total corticosterone			CBG capacity			Free corticosterone		
	df	F stat	F pr	df	F stat	F pr	df	F stat	F pr
Time after implantation	5, 438.8	29.83	<0.001	5, 308.2	1.44	0.210	5, 320.6	14.37	<0.001
Treatment group	1, 433.5	7.34	0.007	1, 73.7	0.02	0.896	1, 78.2	1.26	0.265
Time after implantation x Treatment group	5, 429.7	33.04	<0.001	5, 314.5	2.05	0.071	5, 323.5	15.78	<0.001

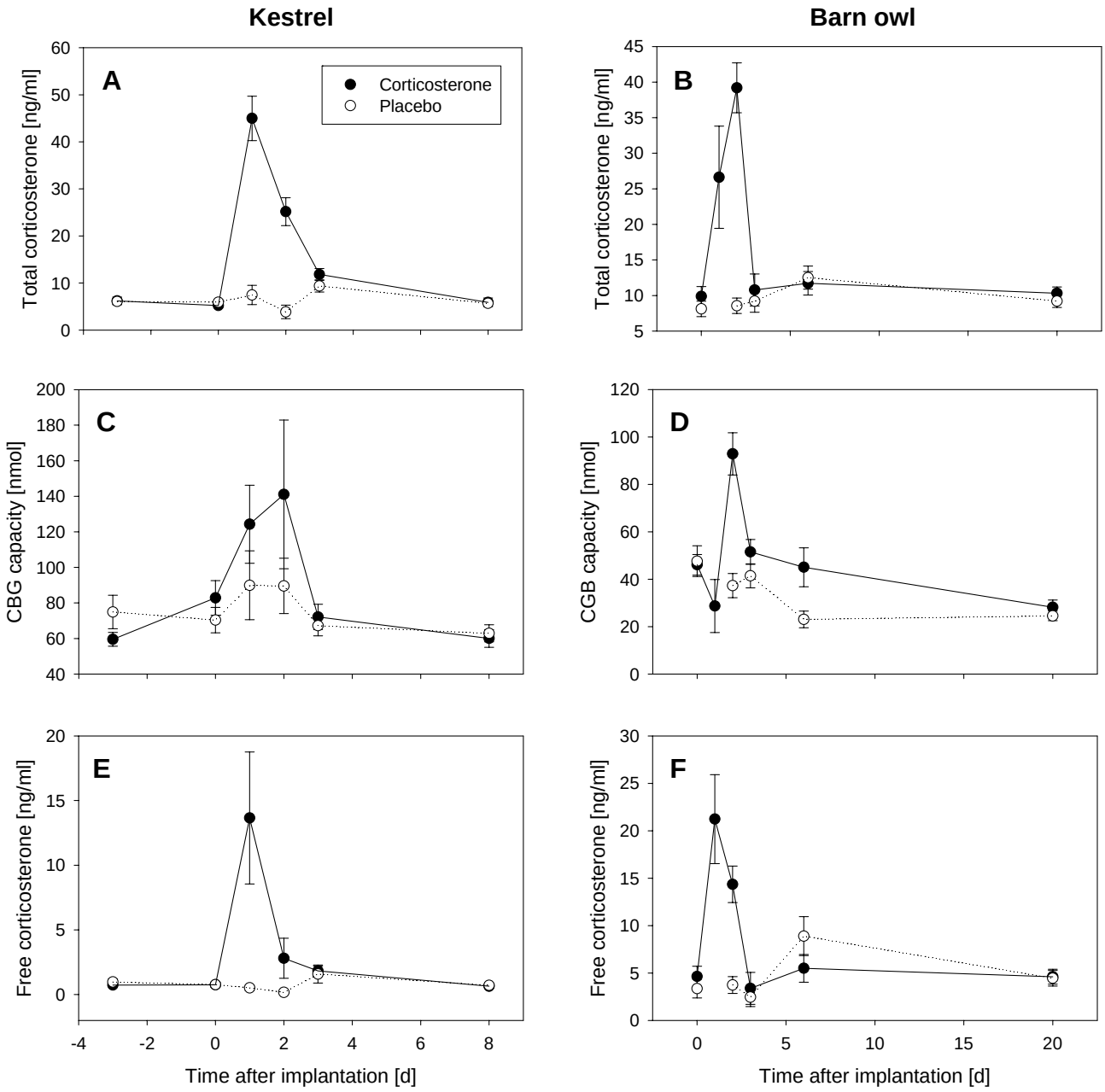

Fig. 2. Effect of corticosterone-releasing pellets on baseline plasma concentration of total corticosterone (A and B), on CBG capacity (C and D) and on estimated plasma concentration of free corticosterone (E and F) of kestrel (left column) and barn owl nestlings (right column). Indicated are means ± SE of a sample size of 36 to 55 kestrel and 21 to 79 barn owl nestlings per time point and treatment group (cort- and placebo-nestlings). Exceptions in sample size are day 1 and 2 after implantation in the kestrel with only 4 - 9 samples per treatment group, day 1 after implantation in the barn owl with only 5 cort-nestlings and day 3 in barn owls with only 14 samples per treatment group. The pellet was implanted on day 0, which corresponded to an age of 13 days in kestrels and 27 days in barn owls.

implantation (post-hoc test: $F = 39.14$, d.f. = 1, 10.4, $p < 0.001$, Fig. 2A, Fig. 3A, range: 29.78 - 63.80 ng/ml), and tended towards significance 2 days after implantation ($F = 7.92$, d.f. = 1, 4.6, $p = 0.037$, not significant at the Bonferroni-corrected p-level of 0.013); corticosterone levels were indis-

Table 2. Effect of corticosterone pellets on plasma levels of total baseline corticosterone, CBG capacity and estimated free corticosterone in barn owl nestlings. *Treatment group* refers to 96 nestlings implanted with a corticosterone pellet and 91 nestlings implanted with a placebo pellet. *Time after implantation* refers to blood samples taken at day 0 (n = 144), 2 (n = 92), 3 (n =28), 6 (n = 47) or 20 days (n = 78) after implantation (389 measurements in total). The results from a mixed model analysis are given with nestling identity nested in broods as random factors.

	Total corticosterone			CBG capacity			Free corticosterone		
	df	F stat	F pr	df	F stat	F pr	df	F stat	F pr
Time after implantation	4, 362.3	28.50	<0.001	4, 270.1	10.52	<0.001	4, 355.2	7.99	<0.001
Treatment group	1, 367.0	46.47	<0.001	1, 113.7	18.59	<0.001	1, 339.4	9.19	0.003
Time after implantation x Treatment group	4, 345.8	28.39	<0.001	4, 288.8	10.15	<0.001	4, 325.5	8.10	<0.001

tinguishable from those of placebo-nestlings 3 and 8 days after implantation. In barn owl nestlings, total corticosterone in cort-nestlings was significantly increased 2 days after implantation ($F = 66.78$, d.f. = 1, 80.0, $p < 0.001$, Fig. 2B, Fig 3B, range 2 days after implantation: 8.11 - 118.03 ng/ml) and returned to the level of placebo-nestlings 3, 6 and 20 days after implantation ($p > 0.161$). The five values of cort-nestlings 1 day after implantation (range 10.7 – 48.1 ng/ml) indicated that levels were not higher than 2 days after implantation.

CBG capacity in kestrel nestlings did not significantly vary with time or treatment (Table 1), and CBG capacity was not significantly elevated on day 1 and 2 after cort-implantation (Fig. 2C; 1 day: $F = 0.80$, d.f. = 1, 13.0, $p = 0.386$, day 2: $F = 1.58$, d.f. = 1, 2.3, $p = 0.335$). In barn owl nestlings, there was an overall effect of treatment and time and their interaction on CBG capacity (Table 2, Fig. 2D). CBG capacity of barn owl cort-nestlings was significantly elevated 2 days after implanta-

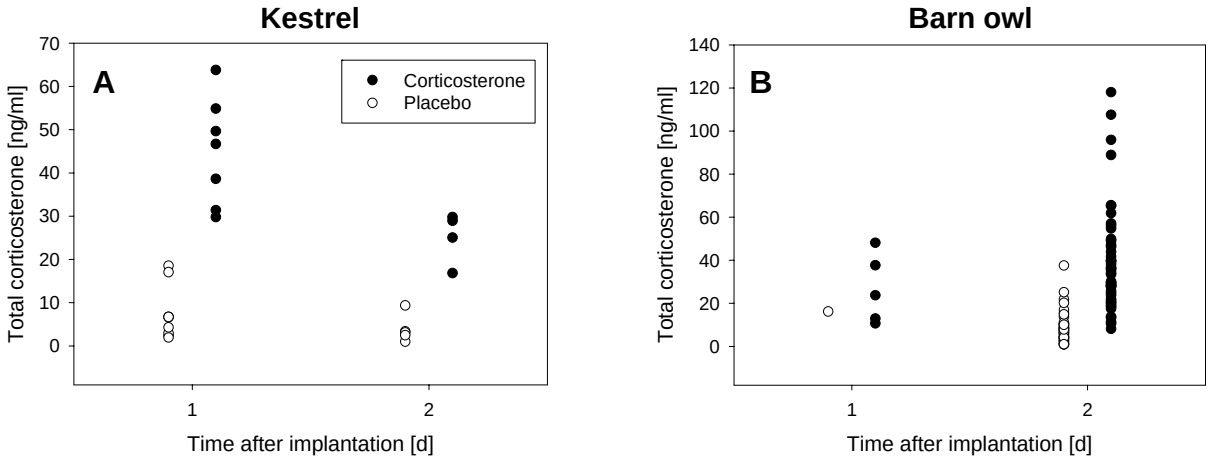

Fig. 3. Individual variation of baseline circulating total corticosterone levels during the two days after pellet implantation in A) kestrel and B) barn owl nestlings. All data points are from different individuals.

tion (F = 37.13, d.f. = 1, 69.7, p < 0.001) and indistinguishable from placebo-nestlings 3, 6 and 20 days after implantation.

Estimated free corticosterone levels varied with time and treatment group depending on time after implantation in both species (Table 1 and 2). There was no difference between the treatment groups before implantation in both species (Fig. 2E, 2F). In kestrels, although the interaction term time after implantation x treatment was highly significant (Table 1), the Bonferroni-corrected post-hoc tests indicated just no significant difference between cort-nestlings and placebo-nestlings one day after implantation (F = 5.33, d.f. = 1, 13.0, p = 0.038, Fig. 2E, not significant at the Bonferroni corrected p-level of 0.013), and no difference between the treatment groups 2, 3 and 8 days after implantation (p = 0.236, 0.847 and 0.072 respectively). In barn owl nestlings, free corticosterone of cort-nestlings was significantly elevated two days after implantation, (F = 26.06, d.f. = 77.8, p < 0.001), while 3, 6 and 20 days after implantation there was no difference between the treatment groups (p = 0.657, 0.153 and 0.733 respectively, Fig. 2F).

3.2. Effect of corticosterone pellets on handling-induced total and free corticosterone levels and CBG capacity

In both species, total and free corticosterone levels depended on time after implantation, treatment group and blood sample (baseline *versus* handling-induced) (interaction time x treatment group x blood sample p < 0.001, Table 3).

Placebo-nestlings of both species showed a marked increase of total plasma corticosterone levels as a response to handling. In cort-nestlings 2 days after implantation (barn owl, Fig. 4B), when

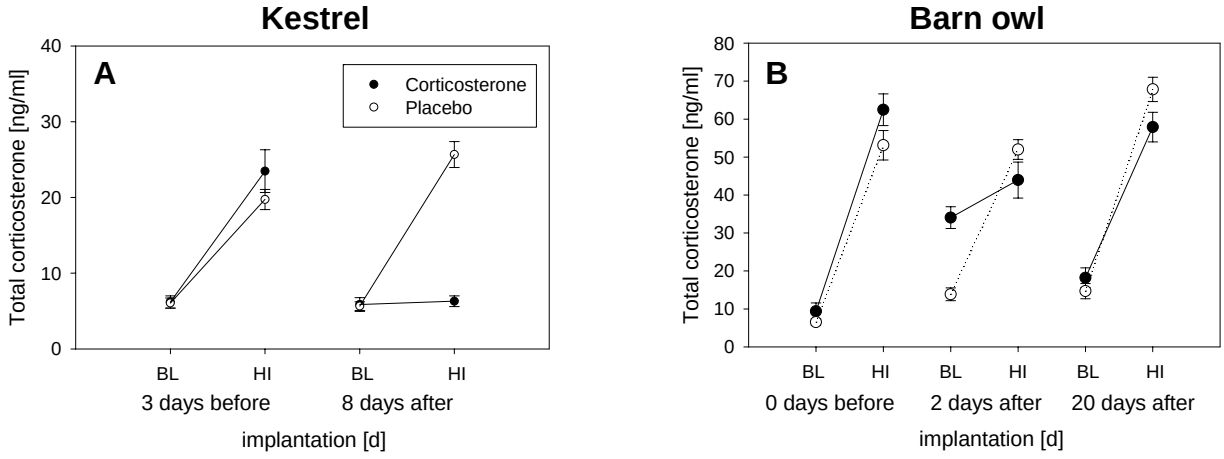

Fig. 4. Baseline (BL) and handling-induced (HI) plasma levels of total corticosterone in A) kestrel and B) barn owl nestlings before and 8 d or 2 and 20 d after implantation of a corticosterone-releasing pellet. Baseline samples were taken within 3 min after capture and handling-induced samples after a mean of 17 min in the kestrel and 19 min in the barn owl. Indicated are means ± SE of a sample size of 36 – 51 kestrel and 26 – 81 barn owls nestlings per time point and treatment group (cort- and placebo-nestlings). The pellet was implanted on day 0, which corresponded to an age of 13 days in kestrels and about 27 days in barn owls.

Table 3. Effect of corticosterone pellets on total baseline and handling induced corticosterone levels in kestrel and barn owl nestlings. *Treatment group* refers to nestlings implanted with a corticosterone or a placebo pellet. *Time after implantation* in the kestrel refers to blood samples taken 3 days before implantation (n = 106) and 8 days after implantation (n = 103) and, in the barn owl, to blood samples taken at day 0 (n = 75), and 2 (n = 158) or 20 days (n = 51) after implantation. *Blood sample* refers to the baseline sample (taken within 3 min) and the handling-induced sample (taken on average 17 (kestrel) or 19 min (barn owl) after capture). The results from a mixed model analysis are given with nestling identity nested in broods as random factors.

	Kestrel			Barn owl		
	Total corticosterone					
	df	F stat	F pr	df	F stat	F pr
Time after implantation	1, 321.7	7.50	0.007	2, 430.2	8.71	<0.001
Blood sample	1, 313.9	155.47	<0.001	1, 395.1	367.96	<0.001
Treatment group	1, 82.6	18.32	<0.001	1, 122.9	3.45	0.066
Time after implantation x Blood sample	1, 313.9	9.67	0.002	2, 396.1	23.05	<0.001
Time after implantation x Treatment group	1, 322.9	39.94	<0.001	2, 476.5	1.15	0.319
Blood sample x Treatment group	1, 313.9	10.75	0.001	1, 395.2	20.90	<0.001
Time after implantation x Blood sample x Treatment group	1, 314.0	29.15	<0.001	2, 396.2	8.25	<0.001

baseline total corticosterone levels were elevated, plasma corticosterone levels increased only little as a response to handling and reached similar levels as in placebo-nestlings (treatment: F = 2.33, d.f. = 1, 120.8, p = 0.130). In cort-nestlings 8 days after implantation (kestrel, Fig. 4A), when baseline levels were low again, the adrenocortical response to handling was virtually absent (interaction time after implantation x treatment group x blood sample p < 0.001, Table 3, Fig. 4A). In barn owl nestlings 20 days after implantation, when baseline levels were similar in both treatment groups, cort-nestlings reached similar handling-induced levels as the placebo-nestlings (F = 3.99, d.f. = 1, 37.6, p = 0.053, Fig. 4B).

4. Discussion

4.1. Effect of corticosterone pellets on total baseline corticosterone levels

The self-degradable corticosterone pellets clearly increased circulating total baseline corticosterone levels in kestrel and barn owl nestlings 1 to 3 days after implantation compared with placebo-nestlings. With the 10 mg and 15 mg 7-day release pellets used in this study, the increase in corticosterone levels was within the range reached by handling before the treatment (kestrel nestlings 8

– 50 ng/ml after 17 min; barn owl nestlings 10 - 110 ng/ml after 19 min) and therefore, within the physiological range. Although the increase in corticosterone levels induced by the pellets varied between individuals, they were clearly higher than in placebo-nestlings (Fig. 3A and B). However, baseline corticosterone levels were not elevated to a constant level, but peaked 1 - 2 days after implantation, dropped to a lower level 2 days after implantation (in the kestrel) and had almost reached placebo-levels 3 days after implantation. Thus, with these pellets, corticosterone levels were elevated for a shorter period than the 7 days indicated by the provider and similar findings were obtained in house sparrows (C.W.B. unpubl. data). There are two possible explanations. First, the corticosterone contained in the pellet may have been released very fast, resulting in peak-like levels higher than intended. It is possible, that the pellet matrix is metabolized faster and corticosterone released in a shorter time period in birds than in mammals, for which the pellet was designed originally. A second explanation is that through the internal negative feedback the release of endogenous corticosterone was strongly reduced until the pellet was metabolized. The finding that the glucocorticoid response to handling was strongly reduced up to 8 days after implantation may suggest that the negative feedback mechanism was indeed in operation (see below). Future studies, using pellets with labelled corticosterone, are needed to determine the relative amounts of endogenous and exogenous corticosterone in the blood and, thus, the importance of the internal negative feedback mechanism to regulate plasma levels of corticosterone after external administration.

In a study implanting silastic tubes filled with corticosterone in starlings *Sturnus vulgaris* and black-legged kittiwakes *Rissa tridactyla*, plasma corticosterone levels also had decreased to near placebo levels 3 or 5 days after implantation (Romero et al., 2005; Angelier et al., 2007), also indicating a potential negative feedback regulation reducing the adrenocortical corticosterone release after some days. Studies using other techniques to administer corticosterone unfortunately do not present the time course of circulating corticosterone after administration. Usually, the resulting circulating corticosterone levels were measured only once at varying time points after the beginning of administration, which prevents the comparison of the time course of corticosterone levels between different methods.

The variation between individuals in baseline corticosterone levels 1 – 2 days after implantation was quite large (Fig. 3). Similar data from published studies using other ways of administering corticosterone are not known to us. It is well established that baseline and handling-induced corticosterone levels (not induced by implants) vary considerably between individuals (e.g. Cockrem and Silverin 2002), hence there is considerable individual variation in the functioning of the HPA-axis that may also hold when administering corticosterone. We found that individual variation in corticosterone levels after implantation in barn owls depends on environmental conditions (Almasi et al, submitted) and are correlated with a genetically determined colour trait (Almasi et al., unpubl. data). Hence, the ability to buffer administered corticosterone seems to be an interesting individual trait in itself.

4.2. Effect of corticosterone pellets on CBG capacity and free baseline corticosterone levels

Concurrent to the increased corticosterone levels, the pellets also increased CBG capacity in barn owl nestlings and tended to increase CBG capacity (small sample size) in kestrel nestlings. This corresponds to increased plasma CBG in house sparrows *Passer domesticus* and serum CBG in mouse pups after glucocorticoid administration (Zhao et al., 1997; Breuner et al., 2003b), but is in contrast to a decrease in CBG production and secretion in rats after glucocorticoid administration (Feldman et al., 1979). It is possible, that the increase in CBG capacity serves to protect partly from the deleterious interferences of high free corticosterone levels with postnatal morphological and cognitive development (Kitaysky et al., 2003, Müller et al., 2009). However, the course of free baseline corticosterone correlated strongly with total baseline corticosterone levels, and the peak in total corticosterone concentration after pellet implantation was buffered only to a small degree by the simultaneously elevated CBG capacity. We did not find any impact of the corticosterone pellets on CBG capacity after the peak of circulating baseline corticosterone.

4.3. Effect of corticosterone pellets on the adrenocortical response to handling

There was only a small adrenocortical response to handling two days after corticosterone pellet implantation in barn owl nestlings and virtually no adrenocortical response to handling 8 days after corticosterone pellet implantation in kestrel nestlings. This can be explained by the negative feedback mechanism controlling circulating corticosterone levels (e.g. Keller-Wood and Dallman, 1984; McEwen et al., 1986). The negative feedback can occur as a rate-sensitive fast feedback and a level-sensitive delayed feedback (Dallman and Yates, 1969). The delayed feedback begins approximately 30 min following glucocorticoid elevation and extends for days. The duration of the subsequent inhibition of the axis depends on the absolute concentration of the steroids, thus may extend beyond the period of hormone administration (e.g. Abe and Critchlow, 1980; Sapolsky et al., 1986).

An attenuated adrenocortical response to an acute stressor, as we observed 2 days after implantation in barn owls, occurs when the animal is already under chronic stress as provoked here by corticosterone administration. As a result of a rate-sensitive fast feedback the hypothamalo-pituitary-adrenal axis shuts down and is not capable of mounting a response to an acute stress (Romero, 2004). One example in free-living birds is the absence of any further corticosterone elevation to capture and handling in a seabird, when a severe storm had already substantially increased circulating corticosterone (Smith et al., 1994). The absence of an adrenocortical response to handling in kestrel nestlings eight days after corticosterone pellet implantation (when baseline total corticosterone levels were not elevated anymore) may be explained by an extension of a level-sensitive delayed feedback. Alternatively, it may be explained by the continuous release of corticosterone from the pellet provoking the shut-down of the hypothamalo-pituitary-adrenal axis, as mentioned above.

Twenty days after corticosterone pellet implantation, the HPA axis seemed to have recovered from the corticosterone manipulation. As a response to handling barn owl cort-nestlings showed a similar increase in circulating total corticosterone levels as placebo-nestlings.

Conclusions

Self-degradable pellets are an efficient tool to artificially elevate corticosterone levels with one intervention. The dose and duration-dependent effects of corticosterone require the monitoring of the resulting circulating corticosterone levels. It is to be expected, that the release from pellets, and possibly from other implants, is not constant over time or that a delayed feedback sets in after a few days which both result in different plasma levels, and thus possibly different effects, of corticosterone at different stages during the experiment. The plasma levels resulting from corticosterone administration can be monitored in a subgroup within the experiment, in an additional group of implanted control animals or in a preliminary study. However, since the HPA axis can vary with season, life history stage, environmental conditions, age and sex (e.g. Wingfield et al., 1994; Kitaysky et al., 1999; Romero, 2002; O'Reilly and Wingfield, 2003; Love and Williams, 2008), any additional group should closely match the experimental groups and live under similar conditions and at the same time or season. Indeed, there is individual variation in corticosterone levels as a response to pellet implantation depending on baseline levels (own unpublished data) and nutritional conditions (Almasi et al., submitted).

When using self-degradable corticosterone pellets, no alcohol or similar solvent should be used during implantation, because even if seemingly evaporated, alcohol can strongly increase the release of corticosterone from the pellet. We also recommend to carefully determine the dosage of the pellet in test birds to avoid pharmacological levels during the first days after implantation.

The interactions of exogenous corticosterone with the negative feedback mechanism and the response to an acute stressor have to be taken into account in future studies. An increase in CBG capacity provoked by corticosterone administration results in an attenuated increase in circulating free baseline corticosterone levels compared with total levels, which may modify the effects of corticosterone on the animal.

Acknowledgements

We warmly thank Ueli Schaffner, Renate Wullschleger, Martin Fischer, Martin Gassner, Linda Bischoff, Christophe Bonetti, Annette Sauter, Jodok Guntern, Matthias Ernst, Lena Senn, Andreas Rieser, Sonja Braaker, Annick Morgenthaler, Ester Pellegrini, Juliette Jullierat, Silvan Rüttimann, Pascal König, Martin Amrein, Silvain Antoniazza, Deborah Ramseier, Silvan Bissegger and Kim Stier for their help in the field. Marc Kéry gave valuable statistical advice. Financial support for this

study was provided by the Swiss National Science Foundation Grant 3100A0-104134 to L.J and S.J-E and PPOOAO-102913 to A.R.

References

Abe, K., Critchlow, V., 1980. Delayed Feedback Inhibition of Stress-Induced Activation of Pituitary-Adrenal Function: Effects of Varying Dose, Rate and Duration of Corticosterone Administration and of Telencephalon Removal. Neuroendocrinology 31, 349-354.

Almasi, B., Roulin, A., Jenni-Eiermann, S., Breuner, C.W., Jenni, L., submitted. Regulation of free corticosterone and CBG capacity under different environmental conditions.

Angelier, F., Clément-Chastel, C., Gabrielsen, G.W., Chastel, O., 2007. Corticosterone and time-activity budget: An experiment with Black-legged kittiwakes. Hormones and Behavior 52, 482-491.

Barsano, C.P., Baumann, G., 1989. Simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria; or how to calculate bound and free hormone? Endocrinology 124, 1101-1106.

Bonier, F., Martin, P.R., Wingfield, J.C., 2007. Maternal corticosteroids influence primary offspring sex ratio in a free-ranging passerine bird. Behavioral Ecology 18, 1045-1050.

Bourgeon, S., Raclot, T., 2006. Corticosterone selectively decreases humoral immunity in female eiders during incubation. Journal of Experimental Biology 209, 4957-4965.

Breuner, C.W., Greenberg, A.L., Wingfield, J.C., 1998. Noninvasive Corticosterone Treatment Rapidly Increases Activity in Gambel's White-Crowned Sparrows (*Zonotrichia leucophrys gambelii*). General and Comparative Endocrinology 111, 386-394.

Breuner, C.W., Orchinik, M., Hahn, T.P., Meddle, S.L., Moore, I.T., Owen-Ashley, N.T., Sperry, T.S., Wingfield, J.C., 2003a. Differential mechanisms for regulation of the stress response across latitudinal gradients. American Journal of Physiology 285, R594-R600.

Breuner, C.W., Wada, H., Shyu, J., Love, O.P., 2003b. Corticosteroid binding globulin capacity responds to chronic hormone treatment but not acute stressors. Integrative and Comparative Biology 46, 1013.

Dallman, M.F., Yates, F., 1969. Dynamic assymmetries in the corticoid feedback path and distribution-metabolism-binding elements of the adrenocortical system. Annals of the New York Academy of Sciences 156, 696-721.

Donker, R.A., Beuving, G., 1989. Effect of corticosterone infusion on plasma corticosterone concentration, antibody production, circulating leukocytes and growth in chicken lines selected for humoral immune responsiveness. British Poultry Science 30, 361-370.

Feldman, D., Mondon, C.E., Horner, J.A., Weiser, J.N., 1979. Glucocorticoid and estrogen regulation of corticosteroid-binding globulin production by rat liver. American Journal of Physiology – Endocrinology and Metabolism 237, E493-E499.

French, S.S., McLemore, R., Vernon, B., Johnston, G.I.H., Moore, M.C., 2007. Corticosterone modulation of reproductive and immune systems trade-offs in female tree lizards: long-term corticosterone manipulations via injectable gelling material. Journal of Experimental Biology 210, 2859-2865.

Hiebert, S.M., Ramenofsky, M., Salvante, K., Wingfield, J.C., Gass, C.L., 2000. Noninvasive Methods for Measuring and Manipulating Corticosterone in Hummingbirds. General and Comparative Endocrinology 120, 235-247.

Horton, B.M., Long, J.A., Holberton, R.L., 2007. Intraperitoneal delivery of exogenous corticosterone via osmotic pump in a passerine bird. General and Comparative Endocrinology 152, 8-13.

Hull, K.L., Cockrem, J.F., Bridges, J.P., Candy, E.J., Davidson, C.M., 2007. Effects of corticosterone treatment on growth, development, and the corticosterone response to handling in young Japanese quail (*Coturnix coturnix japonica*). Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 148, 531-543.

Keller-Wood, M.E., Dallman, M.F., 1984. Corticosteroid inhibition of ACTH secretion. Endocrine Reviews 5, 1-24.

Kitaysky, A.S., Kitaiskaia, E.V., Piatt, J.F., Wingfield, J.C., 2003. Benefits and costs of increased levels of corticosterone in seabird chicks. Hormones and Behavior 43, 140-149.

Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 1999. Dynamics of food availability, body condition and physiological stress response in breeding Black-legged Kittiwakes. Functional Ecology 13, 577-584.

Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 2001. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. Behavioral Ecology 12, 619-625.

Loiseau, C., Sorci, G., Dano, S., Chastel, O., 2008. Effects of experimental increase of corticosterone levels on begging behavior, immunity and parental provisioning rate in house sparrows. General and Comparative Endocrinology 155, 101-108.

Love, O.P., Williams, T.D., 2008. Plasticity in the adrenocortical response of a free-living vertebrate: The role of pre- and post-natal developmental stress. Hormones and Behavior 54, 496-505.

McEwen, B.S., de Kloet, E.R., Rostene, W., 1986. Adrenal Steroid Receptors and Actions in the Nervous System. Physiological Reviews 66, 1121-1188.

Müller, C., Jenni-Eiermann, S., Jenni, L., 2009. Effects of a short period of elevated circulating corticosterone on postnatal growth in free-living Eurasian kestrels *Falco tinnunculus.* Journal of Experimental Biology 212: 1405-1412.

Munro, C., Stabenfeldt, G., 1984. Development of a microtitre plate enzyme immunoassay for the determination of progesterone. Journal of Endocrinology 101, 41-49.

Munro, C.J., Lasley, B.L., 1988. Non-radiometric methods for immunoassay of steroid hormones, in: B.D. Albertson, F.P. Haseltine (Eds.), Non-radiometric assays : technology and application in polypeptide and steroid hormone detection. Alan R. Liss, Inc, cop., New York, pp. 289-329.

O'Reilly, K.M., Wingfield, J.C., 2003. Seasonal, age, and sex differences in weight, fat reserves, and plasma corticosterone in Western Sandpipers. Condor 105, 13-26.

Pravosudov, V.V., 2003. Long-term moderate elevation of corticosterone facilitates avian food-caching behaviour and enhances spatial memory. Proceedings of the Royal Society of London - Series B: Biological Sciences 270, 2599-2604.

Remage-Healey, L., Romero, L.M., 2002. Corticosterone and insulin interact to regulate plasma glucose but not lipid concentrations in molting starlings. General and Comparative Endocrinology 129, 88-94.

Romero, L.M., 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. General and Comparative Endocrinology 128, 1-24.

Romero, L.M., 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology and Evolution 19, 249-255.

Romero, L.M., Strochlic, D., Wingfield, J.C., 2005. Corticosterone inhibits feather growth: Potential mechanism explaining seasonal down regulation of corticosterone during molt. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 142, 65-73.

Sapolsky, R.M., Krey, L.C., McEwen, B.S., 1986. The adrenocortical axis in the aged rat: Impaired sensitivity to both fast and delayed feedback inhibition. Neurobiology of Aging 7, 331-335.

Silverin, B., 1986. Corticosterone-binding proteins and behavioral effects of high plasma levels of corticosterone during the breeding period. General and Comparative Endocrinology 64, 67-74.

Smith, G.T., Wingfield, J.C., Veit, R.C., 1994. Adrenocortical response to stress in the common diving petrel, *Pelecanoides urinatrix*. Physiological Zoology 67, 526-537.

Sokal, R.R., Rohlf, F.J., 2000. Biometry. W. H. Freeman and company, New York.

Wingfield, J.C., Deviche, P., Sharbaugh, S., Astheimer, L.B., Holberton, R., Suydam, R., Hunt, K., 1994. Seasonal changes of the adrenocortical responses to stress in redpolls, *Acanthis flammea*, in Alaska. Journal of Experimental Zoology 270, 372-380.

Wingfield, J.C., Silverin, B., 1986. Effects of corticosterone on territorial behavior of free-living male song-sparrows, *Melospiza melodia*. Hormones and Behavior 20, 405-417.

Zhao, X.F., Scrocchi, L.A., Hammond, G.L., 1997. Glucocorticoids induce corticosteroid-binding globulin biosynthesis by immature mouse liver and kidney. The Journal of Steroid Biochemistry and Molecular Biology 60, 163-169.

Chapter 2

Effects of a short period of elevated circulating corticosterone on postnatal growth in free-living Eurasian kestrels *Falco tinnunculus*

Claudia Müller, Susanne Jenni-Eiermann and Lukas Jenni

Journal of Experimental Biology (2009) 212: 1405 - 1412

Abstract

Environmental conditions affect growth and development and, through developmental plasticity, create phenotypic variation. In suboptimal conditions current survival is traded-off against development. Corticosterone, the main glucocorticoid in birds, plays a major role in this trade-off by reallocating energy from growth to maintenance, but its effect on growth has rarely been investigated in altricial birds under natural conditions in the wild. In free-living Eurasian kestrels *Falco tinnunculus* nestlings, we artificially elevated corticosterone to stress-induced levels during 2 - 3 days in the middle of the nestling stage by implanting self-degradable implants, controlling the treatment with a placebo group. We measured the length of primary feather 8, hand length, tarsus length, body mass and subcutaneous fat stores from day 10 to 25. During corticosterone elevation, primary growth of cort-nestlings was significantly reduced to 71 %, hand and tarsus growth were significantly reduced to 14 % and 26 % respectively, and body mass increase stopped, while subcutaneous fat-store growth was only slightly (non-significantly) reduced. During the following five days, primary growth was still significantly suppressed to 84 %, while hand, tarsus and body-mass growth was back to normal. During the subsequent four days, cort-nestlings compensated the lag in body mass partly by significantly accelerating body mass increase compared with placebo-nestlings. Before fledging, primary length was 10 % shorter, hand and tarsus 5 % and 4 % shorter and body mass 8.5 % lower in cort-nestlings than in placebo-nestlings, while fat score did not differ significantly between the two groups. We showed in free-living, altricial nestlings that a few days of elevated plasma levels of corticosterone alone, without food restriction, suppressed growth which could only partly be compensated afterwards and delayed fledging. Feather, bone and body mass growth were reduced to different degrees, indicating that corticosterone had a differential effect on different structures. This demonstrates that corticosterone is involved in the control of developmental plasticity.

1. Introduction

Growth and development of animals generally are influenced by environmental conditions which, additional to genetic variation, create variation in phenotypes, the material on which selection acts (e.g. Stearns and Hoekstra, 2005). Energetic restrictions or other environmental perturbations during growth and development may provoke a trade-off between current survival and development. Many effects of environmental factors on growth and development are irreversible and thus often have consequences for life (Lindström, 1999).

Glucocorticoids may play an important role in this trade-off between maintenance and development. Across all vertebrate taxa, the activation of the hypothalamo-pituitary-adrenal axis, leading to a rise in glucocorticoids, helps an animal to redirect the available energy and the behaviour from normal activities into a survival mode, to cope with the critical situation (e.g. Wingfield et al., 1998). Elevated glucocorticoid levels inhibit anabolic processes including growth, suppress parts of the immune system and influence appetite (e.g. Bray, 1993; Sapolsky et al., 2000; Lin et al., 2006). Thus environmental factors may affect growth and development directly (e.g. limited nutrients) and indirectly through glucocorticoids which may have suppressing effects on growth and development, such as on body size, body condition, immune system and cognitive functions (e.g. Davison et al., 1983; Saino et al., 2003; Kitaysky et al., 2003; Hull et al., 2007). Therefore, glucocorticoids, as mediators of environmental conditions and through their indirect effects, may be an important additional factor causing developmental plasticity and thus, through long-term or life-long effects, shape the phenotype (Dufty et al., 2002).

The most common environmental factor affecting growth and development is nutritional restriction (through food shortage, competition with siblings, parasites), but also disease, heat, cold and water shortage can have an effect. Most studies used food restrictions to investigate developmental plasticity as a response to environmental conditions, and thereby studied the combined effects of both the nutrient restriction *per se* and the elevated glucocorticoids. Most studies available on the effects of glucocorticoids during growth and development are from precocial species such as quail and chicken under lab conditions (e.g. Davison et al., 1983; Donker and Beuving, 1989; Hayashi et al., 1994). Studies on the effect of glucocorticoids during growth in altricial chicks and under natural conditions are only a few and focus mainly on behavioural aspects (Kitaysky et al., 2001b; Loiseau et al., 2008; Wada and Breuner, 2008). Precocial chicks forage for themselves and glucocorticoids may have similar effects as in adults, i.e. enhance food searching behaviour (Astheimer et al., 1992; Sapolsky *et al.*, 2000). Altricial nestlings, however, are completely dependent on their parents for food, but also have a HPA axis responsive to stressors (e.g. Love et al., 2003). The function of increased glucocorticoids in altricial nestlings may be (a) to improve energy intake by increased begging and becoming more aggressive against siblings (Kitaysky *et al.*, 2003) and (b) to re-allocate the available energy to the most important processes (e.g. Sapolsky *et al.*, 2000; Hochberg, 2002).

The aim of this study was to investigate the effects of a temporary increase of circulating corticosterone (the glucocorticoid in birds) on growth in an altricial bird species, the Eurasian kestrel *Falco tinnunculus*, in natural conditions in the wild. Because we artificially elevated circulating corticosterone for a few days with implants, we could investigate the effects of corticosterone without the confounding effects of food restriction. In most food restriction studies, glucocorticoid levels have not been measured and thus, it remained unknown what the direct effects of food restriction and what the indirect effects of elevated glucocorticoid levels were, while in some food restriction studies glucocorticoids were measured, but the effects on structural growth were not documented (Kitaysky et al., 1999; Kitaysky et al., 2001a; Pravosudov and Kitaysky, 2006; Strochlic and Romero, 2008).

Contrary to most studies investigating the effects of a long stress period on postnatal development, we were interested in the effects of a short period (2-3 days) with clearly elevated baseline corticosterone levels. We used different growth and body condition measures in order to investigate whether growth of skeletal elements, feathers, body mass and subcutaneous body fat stores was affected differently by elevated corticosterone levels. Food restriction studies demonstrated a hierarchy in resource allocation favouring important structures, such as the nervous system and skeletal structures, at the expense of muscles, digestive system and fat stores (Oyan and Anker-Nilssen, 1996; Schew and Ricklefs, 1998; Moe et al., 2004). However, there are hardly any studies investigating effects of elevated corticosterone on various structures (including body size) and tissues in birds, exceptions investigating internal organs include the precocial chicken and quail (e.g. Lin et al., 2006; Hull *et al.*, 2007).

We investigated effects of elevated circulating corticosterone in the middle of the nestling stage and were therefore able to test for compensatory growth after the corticosterone levels returned back to normal. Food restriction studies showed that backlogs in growth may be partly or fully compensated through a prolongation or acceleration of growth (e.g. Emlen et al., 1991; Negro et al., 1994; Bize et al., 2003; Bize et al., 2006) possibly with associated costs (Metcalfe and Monaghan, 2001). Since this study was done in the wild, we examined whether compensatory growth occurred under natural, rather than *ad libitum*, food conditions. Thus our results are directly relevant to natural populations.

In the kestrel, males are slightly smaller than females and nestlings hatch partially asynchronously. Therefore we also investigated whether elevated corticosterone levels affected growth of the smaller sex or smaller chicks differently from growth of the larger ones.

2. Material and Methods

2.1. Study species and study site

The Eurasian Kestrel is a small raptor species with reversed size-dimorphism. Females incubate the 4 - 6 eggs for 29 days starting after the third egg; the three oldest nestlings within a brood therefore have the same age. Tarsus growth is completed at about day 20, maximum body mass is reached on about day 25. Fledging occurs on day 32 - 39. The field work was carried out in North-western Switzerland in an area of 100 km^2, (7°50'E / 47°25'N), where Eurasian kestrels breed in nest boxes mounted on agricultural buildings in open rural landscapes.

2.2. Experimental corticosterone elevation

The experiment was carried out with 109 nestlings of 13 broods in 2004 and 17 in 2005. There was no difference between the years in all parameters; therefore the year was not included in the analysis.

During both breeding seasons, nest boxes were checked weekly from April onwards and before hatching (from May to June) at 4 day-intervals to determine hatching date. At the age of 13 days, two randomly selected nestlings out of the four oldest within a brood were implanted with a self degradable corticosterone implant (*Innovative Research of America,* Sarasota, Florida, U.S.*,* 10 mg corticosterone, 7-day-release) and called cort-nestlings, and the other two were implanted with a placebo pellet (placebo-nestlings). To monitor the effect of the implant on circulating corticosterone levels, we took baseline blood samples at the age of 10, 13, 16 and 21 days in all nestlings. In a subgroup of the nestlings we took an additional sample at day 14 or 15. Within 3 min after taking nestlings out of the nest box, the alar vein was punctured and about 80 µl blood was sampled with heparinized capillary tubes. Corticosterone levels did not rise significantly within 3 min as response to capture ($F = 3.29$, d.f. = 1, $p = 0.071$). Within 30 min, the blood was centrifuged and the plasma stored in liquid nitrogen in the field and at –20°C once in the laboratory. All methods described in this study were approved by the Cantonal committee for animal research (animal experiment permit n°274 from the Cantonal Veterinarian Office of Baselland).

2.3. Growth and body condition measurements

Nestlings were measured at the age of 10, 13, 16, 21 and 25 days (age of the oldest nestlings of the brood). We refrained from visiting the nests after day 25 to avoid premature fledging. Length of the wing and of primary 8 (second longest primary) were measured to the nearest 0.5 mm. An estimate of the skeletal hand length was obtained by subtracting the length of primary 8 from wing length. Tarsus length was measured to the nearest 0.1 mm with digital calipers. Body mass was determined with a spring balance to the nearest g. As in passerines (Kaiser, 1993), we assessed

the subcutaneous fat stores at the furcula by assigning a fat score ranging from 0 to 4 (0: no visible fat; 1: 1 mm stripe of fat at the bottom of the furcular pit; 2: fat stripes 2-3 mm broad; 3: furcular pit nearly covered with fat (about 75%), 4: furcular pit completely filled with fat). Growth rates were calculated on the basis of the number of hours between measurements and expressed as growth rates per 24 h.

From nest controls at hatching and wing length at day 10, we determined the age difference between the oldest nestlings and their siblings. Between the treated nestlings within a brood, this age difference ranged from 0 to 3 days (0 days: 56 nestlings, 1 day: 43 nestlings, 2 days: 12 nestlings, 3 days: 1 nestling). In 18 % of the sampling days, we were unable to measure the nestlings at the intended day for logistic reasons and did it one day earlier or later. Because there was no significant effect of this anticipation or delay in measurements on growth parameters, we omitted it from the analysis.

At the age of 10 and 13 days, 3 and 0 days before the treatment, there were no significant differences between the future corticosterone and placebo groups in any of the parameters measured (data not shown, Fig. 1).

2.4. Hormone assay

Plasma corticosterone concentration was determined using an enzyme immuno assay. Corticosterone in 5 μl plasma and 195 μl water was extracted with 4 ml dichlormethane, re-dissolved in phosphate buffer and given in triplicates in the enzyme immuno assay. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, Progesterone 0.004%, 18-OH-DOC 0.01%, Cortisol 0.12%, 18-OH-B 0.02% and Aldosterone 0.06%) was 1:8'000. HRP (1:400'000) linked to corticosterone served as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. Plasma pools from chickens with two different corticosterone concentrations were included as internal controls on each plate. If the concentration was below the detection threshold, the determination was repeated with 10 μl plasma. If the concentration was still below the detection threshold, the value of the lowest detectable concentration (1 ng ml^{-1}) was assigned. Intra-assay variation ranged from 4.5 to 10.8 % and inter-assay variation from 9.6 to 17.6 %, depending on the concentration of the internal control and the year of determination.

2.5. Sex determination

Nestlings were sexed with molecular methods by fragment analysis on CHD1W/CHD1Z (Fridolfsson and Ellegren, 1999) using blood cells of blood samples extracted with the QIAamp DNA extraction Kit (Qiagen) in 2004 and after Kawasaki (1990) in 2005. Samples from 2004 were analysed at the Swiss Federal Institute for Forest, Snow and Landscape Research in Birmensdorf,

Switzerland and those from 2005 at the Agroscope Research Station ACW in Wädenswil, Switzerland.

2.6. Statistical analysis

Growth rates were analysed with a mixed model for repeated measurements in Genstat 9.1 (Payne, 2003; Thompson and Welham, 2003). In the fixed model, the effect of the corticosterone treatment on primary, hand and tarsus growth rate and body mass and furcular fat store increase was examined taking into account age (in days), time of day, sex, age difference to the oldest nestling within the brood (in days), brood size, the absolute measure of the parameter of this individual at day 10 and hatching date (Julian date). After the main parameters, biologically relevant interactions between Age, Sex, Age difference within the brood and Treatment were tested. The design of the random model was Brood x Age. The nestling variance component was very small and therefore omitted from the random model.

To assess the effect of corticosterone treatment on growth rates, body size and condition before and during the experiment and just before fledging, the growth rates and absolute morphological measurements (primary 8, hand and tarsus length, body mass, fat score) at day 10, 13, 16, 21 and 25, were analysed separately in post-hoc Mixed Models with time of day, sex, the age difference to the oldest nestling within the brood, brood size, hatching date, treatment and relevant two-way interactions in the fixed model and Brood as random model. Because individuals were measured repeatedly, significance levels were adjusted according to Bonferroni (Sokal and Rohlf, 2000). All model residuals were normally distributed.

3. Results

The self-degradable corticosterone implants elevated circulating baseline corticosterone levels from 5.5 ± 0.40 ng/ml (n = 88, no difference between the future placebo and corticosterone groups) on day 13 before implantation to 45.0 ± 4.7 ng/ml (range 29.7 – 63.8 ng/ml, n = 7) the following day and 25.2 ± 2.96 ng/ml (range 17 – 30 ng/ml, n = 4) on day 15. On day 16, the levels of the corticosterone implanted nestlings (11.8 ± 1.26 ng/ml) were only slightly elevated compared with those of the placebo group, whose corticosterone levels averaged 6.65 ± 1.55 ng/ml from day 14 to 16. Thus, the corticosterone implants clearly elevated circulating corticosterone levels during the middle of the nestling stage for 2 to 3 days (for details see Müller et al., 2009).

Corticosterone treatment occurred when the growth rate of primary 8 was high and temporarily reduced it (interaction age × treatment highly significant, Table 1, Fig. 1a). Primary growth rate in cort-nestlings was significantly reduced (for statistics see Fig. 1) to 71 % of the placebo-nestlings during the period of elevated circulating corticosterone (from nestling day 13 to 16, Fig. 1a). During the five days after treatment (day 16 to 21), primary growth rate was still significantly

Table 1. Effect of corticosterone treatment and other parameters on primary, hand and tarsus growth rates and body mass and fat score increase of Eurasian Kestrel nestlings. 109 nestlings in 30 broods were measured at the age of 10, 13, 16, 21 and 25 days resulting in 428 measurements. Growth rates per nestling were analysed using a repeated measures mixed model.

		Primary growth rate		Hand growth rate		Tarsus growth rate		Body mass increase		Fat score increase	
Explanatory variable	df	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p
Age (of the oldest nestling within the brood) [d]	3	41.09	<0.001	90.78	<0.001	620.90	<0.001	156.59	<0.001	36.46	< 0.001
Time of day [h]	1	2.36	0.124	2.37	0.123	3.46	0.063	14.43	<0.001	0.14	0.706
Sex	1	3.86	0.049	3.02	0.082	2.85	0.091	17.27	<0.001	0.30	0.581
Age difference to oldest nestling [d]	1	2.36	0.124	6.81	0.009	16.58	<0.001	18.86	<0.001	0.01	0.906
Brood size	1	0.37	0.543	0.00	0.982	0.45	0.501	1.44	0.231	0.06	0.803
Measure at day 10 [mm], [g]	1	13.18	<0.001	15.06	<0.001	66.42	<0.001	11.61	<0.001	19.70	< 0.001
Hatching date [d]	1	0.12	0.728	0.01	0.935	0.61	0.436	1.58	0.209	0.76	0.384
Treatment group	1	102.82	<0.001	6.13	0.013	16.97	<0.001	49.44	<0.001	0.58	0.447
Age x Sex	3	3.92	0.271	5.99	0.112	11.04	0.012	3.09	0.377	0.64	0.888
Age x Age difference to oldest nestling	3	2.60	0.457	1.23	0.745	10.70	0.013	10.66	0.014	0.80	0.850
Age x Treatment group	3	85.00	<0.001	34.62	<0.001	105.46	<0.001	360.78	<0.001	8.99	0.029
Sex x Treatment group	1	0.97	0.324	0.46	0.498	0.26	0.609	0.01	0.923	0.01	0.917
Age difference to oldest nestling x Treatment group	1	1.14	0.287	1.28	0.258	0.01	0.928	0.30	0.581	0.06	0.800
Age x Sex x Treatment group	3	0.75	0.860	5.71	0.126	2.50	0.476	6.38	0.094	0.78	0.854
Age x Age difference to oldest nestling x Treatment group	3	3.27	0.352	7.57	0.056	2.37	0.499	2.14	0.544	7.05	0.070

Table 2. Effect of the corticosterone treatment and other parameters on primary, hand and tarsus length, body mass and fat score at day 25 (before fledging) of Eurasian Kestrel nestlings (109 nestlings in 30 broods).

		Primary length		Hand length		Tarsus length		Body mass		Fat score	
Explanatory variable	df	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p
Time of day [h]	1	0.36	0.549	1.84	0.175	6.96	0.008	1.77	0.184	4.60	0.032
Sex	1	4.22	0.040	8.04	0.005	0.07	0.784	36.77	<0.001	0.30	0.585
Age difference to oldest nestling [d]	1	35.26	<0.001	0.01	0.930	4.82	0.028	0.02	0.882	3.48	0.062
Brood size	1	2.33	0.127	0.23	0.635	2.65	0.105	0.41	0.524	0.16	0.693
Hatching date [d]	1	0.00	0.997	0.31	0.579	1.86	0.103	4.45	0.035	1.17	0.279
Treatment group	1	98.90	<0.001	21.89	<0.001	39.78	<0.001	37.94	<0.001	0.33	0.568
Sex x Treatment group	1	1.62	0.203	1.05	0.305	1.02	0.312	0.03	0.859	0.01	0.939
Age difference to oldest nestling x Hatching date	1	4.06	0.044	0.77	0.379	0.13	0.716	2.63	0.105	0.30	0.586
Age difference to oldest nestling x Treatment group	1	1.13	0.289	1.17	0.280	0.03	0.857	0.39	0.531	0.48	0.488

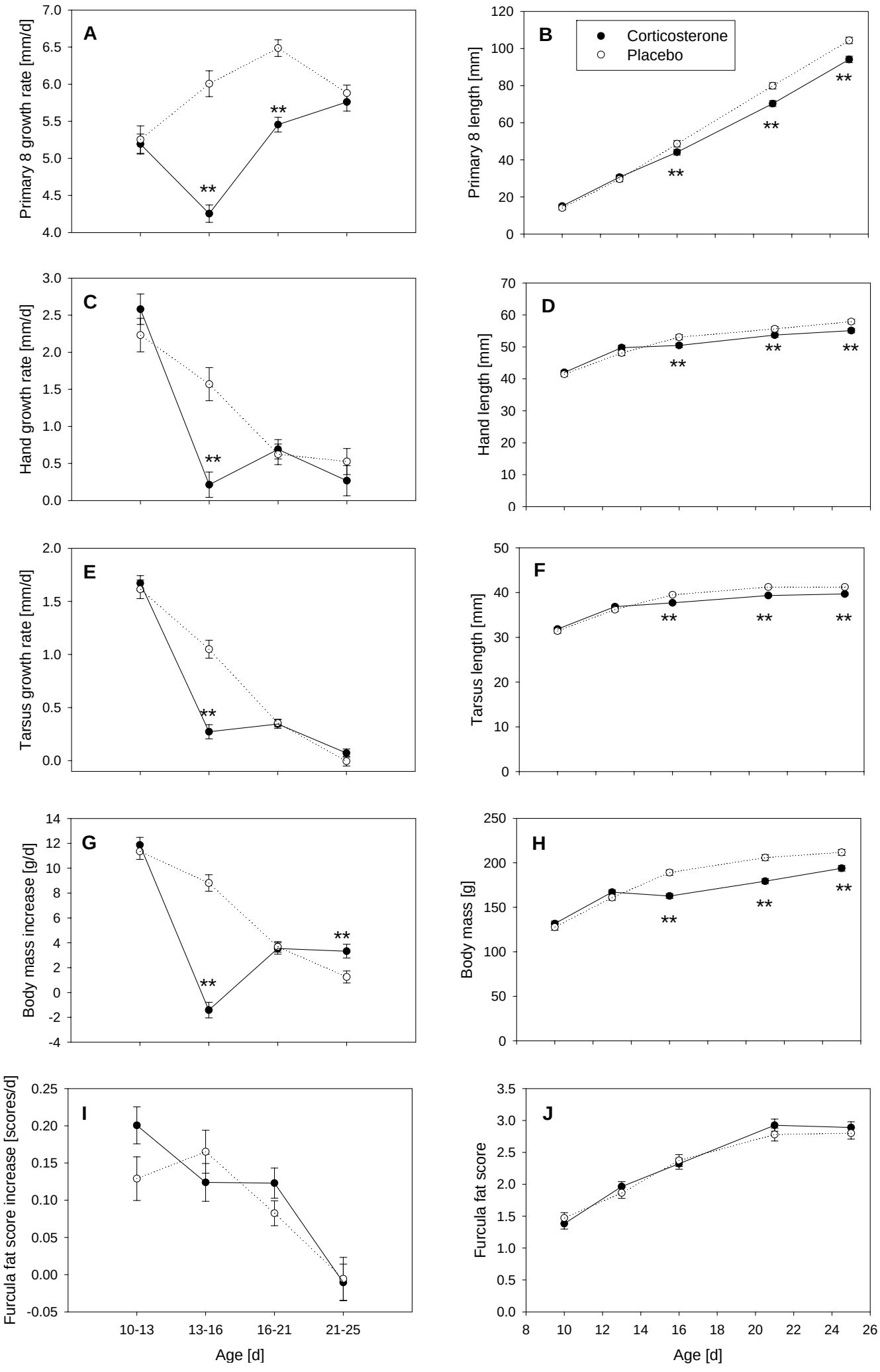
A
Primary 8 growth rate [mm/d]
7.0
6.5
6.0
5.5
5.0
4.5
4.0
**
**
B
Corticosterone
Placebo
Primary 8 length [mm]
120
100
80
60
40
20
0
**
**
**
C
Hand growth rate [mm/d]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
D
Hand length [mm]
70
60
50
40
30
20
10
0
**
**
**
E
Tarsus growth rate [mm/d]
2.0
1.5
1.0
0.5
0.0
**
F
Tarsus length [mm]
50
40
30
20
10
0
**
**
**
G
Body mass increase [g/d]
14
12
10
8
6
4
2
0
-2
-4
**
**
H
Body mass [g]
250
200
150
100
50
0
**
**
**
I
Furcula fat score increase [scores/d]
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
10-13
13-16
16-21
21-25
Age [d]
J
Furcula fat score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
8
10
12
14
16
18
20
22
24
26
Age [d]

Fig. 1. Mean daily growth rates (± SE) (left column) and mean absolute values (± SE) (right column) of the length of primary 8, hand length, tarsus length, body mass and furcular fat score in kestrel nestlings from the age of day 10 to 25. Corticosterone pellets were implanted on day 13 and circulating corticosterone was elevated from day 13 to 16. Asterisks (**) indicate significant differences between cort- and placebo-nestlings (post-hoc mixed models, treatment: $p < 0.001$).

reduced to 84 % of the placebo group and recovered only in the following period from day 21 to 25, then corresponding to the placebo group again. At day 25, cort-nestlings had a significantly shorter primary (10.3 mm or 10 %, Table 2, Fig. 1b) than the placebo-nestlings. At this age, primary 8 had reached about 53 % of its final length.

For both hand and tarsus, corticosterone treatment occurred during the final growth phase as shown by the strongly decreasing growth rates in placebo birds from day 10 to day 21 (Fig. 1c, 1e, age significant, Table 1). Corticosterone treatment temporarily reduced hand and tarsus growth significantly (interaction age × treatment significant, Fig. 1c, 1e). During the period of elevated circulating corticosterone (day 13 to 16), hand growth rate of the cort-nestlings was only 14 % of the rate of the placebo group (Fig. 1c) and tarsus growth rate only 26 % (Fig. 1e). After the period of high corticosterone levels (day 16 to 21), hand and tarsus growth rates of the cort-nestlings had recovered and were indistinguishable from the placebo group. At day 25, when hand growth is almost completed, cort-nestlings had a 2.8 mm or 5 % shorter hand length (Table 2, Fig. 1d). In contrast to the hand, cort-nestlings still grew their tarsi from day 21 to 25 and compensated to a certain degree, while tarsus growth was already completed in the placebo group (Fig. 1e, b). On day 25, cort-nestlings had a 1.5 mm or 4 % shorter tarsus than the placebo group (Table 2, Fig. 1f).

Corticosterone treatment strongly affected body mass growth (Table 1, Fig. 1g). Body mass growth rate of the cort-nestlings was negative during the period of elevated circulating corticosterone while the placebo-nestlings gained about 9 g/day. During the five days after the treatment, from day 16 to 21, both groups had similar growth rates. From day 21 to 25, the body mass retardation of cort-nestlings was partly compensated by growing at 265 % compared with the placebo group (difference in growth rate significant). On day 25, cort-nestlings had a significantly lower body mass (by 18 g or 8.5 %) than the placebo group (Table 2, Fig. 1h). At this age, adult body mass is reached or surpassed by normally developing nestlings.

Corticosterone treatment had no overall significant effect on furcular fat score increase and placebo- and cort-nestlings had about the same furcular fat score on day 25, (Fig. 1i, 1j), but the growth curves of the two groups had slightly different shapes (interaction age × treatment significant, Fig. 1i, Table 1). This may be partly due to the fact that placebo-nestlings by chance had more subcutaneous fat reserves on day 10 than future cort-nestlings and therefore fat was accumulated at a slightly lower rate until day 13 than in cort-nestlings, whose fat scores were lower on day 10.

Despite some significant variation in growth rates with sex, developmental stage and within-brood age differences, corticosterone treatment did not affect growth of the sexes and asynchronously hatched or less developed nestlings differentially in any of the measurements taken (Table 1).

4. Discussion

Our study revealed pronounced effects of a short-term corticosterone treatment on various growth parameters in altricial wild kestrel chicks under natural conditions. These effects were only partly compensated after corticosterone levels returned to normal after three days. By administering corticosterone with implants, we evaluated the effects of increased corticosterone levels on postnatal growth without the confounding effects of a reduced availability of growth substrates caused by food restriction, the most common stressor used in lab studies. In altricial nestlings, glucocorticoids may be released as a response also to stressors not involving food reduction, such as disease, disturbance by predators and humans, interactions with siblings, etc. The self-degradable corticosterone pellets elevated circulating corticosterone to moderate or high levels for three days during the middle of the nestling stage. This is a short period of stress compared with other studies administering corticosterone (e.g. Lin et al., 2006; Hull et al., 2007) or restricting food over weeks in chicks (e.g. Oyan and Anker-Nilssen, 1996; Benowitz-Fredericks et al., 2006). The plasma levels of corticosterone induced by the implants were within levels occurring naturally in the smallest kestrel chicks during nutritional stress (25 - 100 ng/ml) and in kestrel chicks after 20 min of handling (10 – 50 ng/ml; own unpublished data).

Immediate effects of corticosterone on growth

During the period of elevated circulating corticosterone (days 13 - 16 of age), corticosterone treatment affected all growth parameters investigated, but to very different degrees (reduction to 71 % in feather growth rate, to 14 % in hand growth, to 26 % in tarsus growth, to 0 % in body mass growth and no effect on subcutaneous fat stores. Feather growth was only reduced to 71 %, although wing feather growth was at its maximum, and is a very costly and inefficient process with only a 20 % energetic efficiency of feather synthesis in the kestrel (Dietz et al., 1992). Feather growth reduction (to 84 %) extended also into the days when circulating corticosterone had returned to normal levels, as observed in moulting starlings (Romero et al., 2005). Growth of the skeletal elements of all extremities was strongly reduced during the period of elevated corticosterone levels. Contrary to feather growth, growth of the hand and tarsus recovered completely to the (then low) rate of the control group directly after the period with elevated corticosterone levels.

That body mass growth was completely stopped and body mass even reduced under corticosterone treatment agrees with studies in precocial bird species, such as chicken and quail (Davi-

son et al., 1983; Buyse et al., 1987; Donker and Beuving, 1989; Siegel et al., 1989; Bray, 1993; Hayashi et al., 1994; Post et al., 2003; e.g. Dong et al., 2007; Hull et al., 2007). Body mass growth recovered after the treatment period. Because no birds were sacrificed, we cannot explore which parts of the body were most affected by corticosterone treatment, except for growth of feathers and skeletal extremities (see above) and fat stores. Corticosterone treatment did not reduce peripheral body fat stores, demonstrating that nestlings were not in a normal fasting state, such as under food restriction.

Corticosterone treatment thus had a differential effect on growth of different body parts and organs. This hierarchy in growth allocation under elevated circulating corticosterone agrees partly with the hierarchy found in food restriction experiments, indicating a regulating role of corticosterone during food restriction. Structural growth is protected at the expensive of more flexible body tissues such as muscles (Oyan and Anker-Nilssen, 1996; Moe et al., 2004; Benowitz-Fredericks et al., 2006). Feather growth is even more strongly supported than bone growth, similarly as in zebra finch nestlings raised with low-quality food (Boag, 1987). An exception are fat stores which are depleted first under food restriction, but kept under corticosterone treatment; this may be due to the well-known fattening effect of chronic corticosterone administration (e.g. Davison et al. 1983; Buyse et al. 1987). Flight feathers are crucial for flight performance and were in their main growth phase during elevated corticosterone levels, presumably therefore their growth was most buffered. Additionally, hand and tarsus were in their final growth phase during the treatment and had almost reached their definite length. Thus corticosterone could only have a small effect on final size. Corticosterone treatment in another developmental stage may possibly show a different growth allocation.

There may be two mechanisms by which corticosterone reduces feather growth. The first is by interference with the growth hormone – IGF-1-axis (Hochberg, 2002), the primary control of postnatal growth (McNabb et al., 1998). The suppressed growth rate of feathers (consisting to 95 % of protein) can be explained by the inhibition of protein synthesis with high corticosterone levels (Sapolsky *et al.*, 2000). The depressed feather growth rate in the days, when circulating corticosterone levels had returned to the level of the control group, could be the result of products induced by corticosterone on gene transcription that still existed (Sapolsky *et al.*, 2000). Glucocorticoids impair bone growth 1) indirectly with catabolic effects on bone and cartilage protein, interfering with the growth hormone - IGF-1-axis, and by disturbing normal calcium balance, and 2) directly, by impeding anabolic processes at the growth plate and the adjacent tissues of the bones (Hochberg, 2002). As a second mechanism, corticosterone treatment may also have affected growth by reducing appetite (Sapolsky *et al.*, 2000) and possibly a reduced digestive efficiency or increased maintenance energy expenditure as observed in precocial chicks (Dong *et al.*, 2007). However, the evidence of the effects of corticosterone on food intake is controversial. Other studies observed increased or no change in food intake in quail and chicken (Bray 1993, Buyse et al. 1987; Simon 1984; Davison et al. 1983) and increased (Hayashi et al. 1994) or decreased food

conversion (Siegel et al. 1989). Whether kestrel nestlings could increase food intake by increased begging and aggressiveness against siblings (Kitaysky *et al.*, 2003) remains to be shown. Food intake of cort-nestlings during the two days after implantation was not measurably reduced compared with placebo-nestlings (video observations of feeding rates, own unpublished data). This would indicate that elevated corticosterone increased energy expenditure which contributed to the loss of body mass (DuRant et al., 2008).

Compensatory growth

The compensatory growth pattern varied widely between body structures. Accelerated growth occurred only in body mass and to a slight extent in tarsus, while growth of hand and feather just resumed the growth rate of the corresponding age (i.e. the growth rate of the placebo-nestlings). A prolonged growth period occurred probably in body mass and feather length. Primary feather length, measured at day 25, had only reached about 53 % of its final length, the primaries continue to grow after fledging. Therefore, we were unable to assess whether the cort-nestlings prolonged or accelerated primary growth after day 25 when their primary length was 10 % shorter than in placebo-nestlings. Growth of the two skeletal structures were terminated by day 25. Maturation of the tarsus and the hand bone seemed not to be slowed down, preventing a prolonged or accelerated growth to reach a normal tarsus and hand length.

Cort-nestlings did not accelerate body-mass growth rate during the period immediately following that of elevated corticosterone levels, but later before fledging. On day 25, their body mass was only 9 % lower than in placebo-nestlings, compared to a body mass 14 % lower on day 16 and 21, indicating a prolongation of the body mass growth phase. It is possible that the cort-nestlings compensated their lag in body mass further until fledging by prolonging growth or by not reducing body mass just before fledging, as normally developed placebo-nestlings do, similar as in food stressed altricial song sparrows *Melospiza melodia* (Searcy et al., 2004). Since the corticosterone-treated nestlings fledged about two days later than their placebo siblings (own unpublished data), it is likely that they prolonged the build-up of body mass by two days and reached a similar fledging weight as the placebo siblings.

To our knowledge, this is the first study which examined the development of body mass well after artificially elevated corticosterone levels returned back to normal. All other studies known to us stopped at the end of corticosterone administration without monitoring potential compensatory growth. Studies investigating a natural or experimental food restriction in altricial nestlings, which lead to a similar lag in body mass as with our corticosterone treatment, found either a similar restoring of the growth rate to the normal rates of the control group and prolonged growth (Schew, 1995) or accelerated growth (Negro *et al.*, 1994; Bize *et al.*, 2006), so that the backlog was at least partially compensated. The timing and extent of the compensatory growth seems to depend on the severity, the developmental phase and the duration of the nutritional restriction.

Long-term effects of corticosterone on morphology and body condition

Only two days of elevated corticosterone levels resulted in life-long impacts on morphology. Bone growth is completed before fledging and tarsus remained 4 % and hand skeleton 5 % shorter (as in three altricial food restricted species; (Boag, 1987; Schew, 1995; Searcy et al., 2004) and this is irreversible. The consequences of a shorter leg and wing length in the kestrel are unknown. Concerning sexual selection, female kestrels select males with shorter tarsi and do not discriminate short-winged males (Hakkarainen et al., 1996), so that the slightly shorter cort-nestling males are probably not at a disadvantage. However, this might be different in female kestrels, and in species without reversed sexual size-dimorphism, where smaller birds often have a reduced fitness (Richner, 1989).

If wing feathers of cort-nestlings do not fully recover, wing length and wing area would be somewhat smaller, possibly negatively affecting flight performance and hunting capabilities and presumably survival. In male kestrels, however, a smaller wing does not have to be a disadvantage. Short-winged male kestrels are somewhat better hunters than longer-winged ones (Hakkarainen *et al.*, 1996). If fledglings survive the first year, they have the opportunity to replace shorter primaries by longer ones during moult in the next summer. This might be a good strategy, because accelerated feather growth is associated with a lower feather quality (Dawson et al., 2000) and general costs of compensatory growth (Metcalfe and Monaghan, 2001).

Cort-nestlings fledged with similar fat stores as placebo nestlings. However, if retardation in body mass of cort-nestlings was not compensated until fledging, cort-nestlings would have fledged at a lower body mass. Several studies have shown that body mass at fledging is a good predictor of survival (Lindén et al., 1992; Naef-Daenzer et al., 2001). In kestrels, fledglings are still fed by their parents up to 4 weeks after fledging and it is possible, that the corticosterone-treated nestlings could catch up then.

No differential effect of corticosterone on the sexes and ages

In all morphological parameters investigated corticosterone treatment did not differentially affect the sexes and younger nestlings within the brood. Thus, we did not find indications that later-born, smaller nestlings are affected more strongly than older, larger ones. Hence, we have no evidence that elevated corticosterone levels amplify size-dependent mortality as prepared by asynchronous hatching. However, size differences between sexes and ages were not large in our kestrels.

Conclusions

This study demonstrated in altricial and free-living nestlings that elevated plasma levels of corticosterone alone, without food restriction, suppress growth and, thus, that the action of corticosterone alone is involved in the control of developmental plasticity. It follows that environmental stressors

without energetic restrictions (e.g. human disturbance, disease) may have a growth-suppressing effect and consequently the potential to shape the phenotype. Already a relatively short disturbance of 2 - 3 days resulting in high corticosterone levels can have far-reaching consequences on morphology and fitness. In the context of conservation biology, it will be important to investigate the effect of repeated high corticosterone levels, as may occur as a response to repeated human disturbance, on growth and development.

With the corticosterone administered in this study, effective during only a few days, we provoked a reduced primary feather and tarsus length before fledging corresponding to siblings born three days later. However, feather, bone and body mass growth were reduced to different degrees. This indicates that corticosterone has not just an overall suppressing effect on growth, but a differential effect favouring presumably the most sensitive tissues of the actual developmental phase. Such a differential effect was also observed in nutritional restriction experiments. Because food shortage usually results in elevated corticosterone levels, this points to a steering role of corticosterone on growth allocation during nutritional restriction and other disturbances.

Acknowledgements

We warmly thank Ueli Schaffner, Renate Wullschleger, Martin Fischer, Martin Gassner, Linda Bischoff, Christophe Bonetti, Annette Sauter, Jodok Guntern, Matthias Ernst, Lena Senn for their committed help in the field work. We are grateful to Ester Pellegrini and Monika Pfunder, who performed the molecular sex determination at the Swiss Federal Institute for Forest, Snow and Landscape Research in Birmensdorf and at the Agroscope Changins-Wädenswil Research Station ACW in Wädenswil. Marc Kéry gave valuable statistical advice, Marc Kéry, Martin Grüebler and Bettina Almasi commented on an earlier version of the manuscript. Financial support for this study was provided by the Swiss National Science Foundation Grant 3100A0-104134 to L.J and S.J-E.

References

Astheimer, L.B., Buttemer, W.A., Wingfield, J.C., 1992. Interactions of corticosterone with feeding, activity and metabolism in passerine birds. Ornis Scandinavica 23, 355-365.

Benowitz-Fredericks, Z.M., Kitaysky, A.S., Thompson, C.W., 2006. Growth and allocation in captive common murre (*Uria aalge*) chicks. Auk 123, 722-734.

Bize, P., Metcalfe, N.B., Roulin, A., 2006. Catch-up growth strategies differ between body structures: interactions between age and structure-specific growth in wild nestling Alpine Swifts. Functional Ecology 20, 857-864.

Bize, P., Roulin, A., Bersier, L.F., Pfluger, D., Richner, H., 2003. Parasitism and developmental plasticity in Alpine swift nestlings. Journal of Animal Ecology 72, 633-639.

Boag, P.T., 1987. Effects of nestling diet on growth and adult size of zebra finches (*Poephila guttata*). Auk 104, 155-166.

Bray, M.M., 1993. Effect of ACTH and glucocorticoids on lipid metabolism in the Japanese quail, *Coturnix coturnix japonica*. Comparative Biochemistry and Physiology Part A: Physiology 105, 689-696.

Buyse, J., Decuypere, E., Sharp, P.J., Huybrechts, L.M., Kühn, E.R., Whitehead, C., 1987. Effect of corticosterone on circulating concentrations of corticosterone, prolactin, thyroid hormones and somatomedin C and on fattening in broilers selected for high or low fat content. Journal of Endocrinology 112, 229-237.

Davison, T.F., Rea, J., Rowell, J.G., 1983. Effects of dietary corticosterone on the growth and metabolism of immature *Gallus domesticus*. General and Comparative Endocrinology 50, 463-468.

Dawson, A., Hinsley, S.A., Ferns, P.N., Bonser, H.C., Eccleston, L., 2000. Rate of moult affects feather quality: a mechanism linking current reproductive effort to future survival. Proceedings of the Royal Society - B: Biological Sciences 267, 2093-2098.

Dietz, M.W., Daan, S., Masman, D., 1992. Energy requirements for molt in the kestrel *Falco tinnunculus*. Physiological Zoology 65, 1217-1235.

Dong, H., Lin, H., Jiao, H.C., Song, Z.G., Zhao, J.P., Jiang, K.J., 2007. Altered development and protein metabolism in skeletal muscles of broiler chickens (*Gallus gallus domesticus*) by corticosterone. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 147, 189-195.

Donker, R.A., Beuving, G., 1989. Effect of corticosterone infusion on plasma corticosterone concentration, antibody production, circulating leukocytes and growth in chicken lines selected for humoral immune responsiveness. British Poultry Science 30, 361-369.

Dufty, A.M., Clobert, J., Moller, A.P., 2002. Hormones, developmental plasticity and adaptation. Trends in Ecology and Evolution 17, 190-196.

DuRant, S.E., Romero, L.M., Talent, L.G., Hopkins, W.A., 2008. Effect of exogenous corticosterone on respiration in a reptile. General and Comparative Endocrinology 156, 126-133.

Emlen, S.T., Wrege, P.H., Demong, N.J., Hegner, R.E., 1991. Flexible growth rates in nestling white-fronted bee-eaters: a possible adaptation to short-term food shortage. Condor 93, 591-597.

Fridolfsson, A.K., Ellegren, H., 1999. A simple and universal method for molecular sexing of non-ratite birds. Journal of Avian Biology 30, 116-121.

Hakkarainen, H., Huhta, E., Lahti, K., Lundvall, P., Mappes, T., Tolonen, P., Wiehn, J., 1996. A test of male mating and hunting success in the kestrel: The advantages of smallness? Behavioral Ecology and Sociobiology 39, 375-380.

Hayashi, K., Nagai, Y., Ohtsuka, A., Tomita, Y., 1994. Effects of dietary corticosterone and trilostane on growth and skeletal muscle protein turnover in broiler cockerels. British Poultry Science 35, 789-798.

Hochberg, Z., 2002. Mechanisms of Steroid Impairment of Growth. Hormone Research 58, 33-38.

Hull, K.L., Cockrem, J.F., Bridges, J.P., Candy, E.J., Davidson, C.M., 2007. Effects of corticosterone treatment on growth, development, and the corticosterone response to handling in

young Japanese quail (*Coturnix coturnix japonica*). Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 148, 531-543.

Kaiser, A., 1993. A new multi-category classification of subcutaneous fat deposits of songbirds. Journal of Field Ornithology 64, 246-255.

Kawasaki, E.S., 1990. Sample preparation from blood, cells, and other fluids. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols - A guide to methods and applications. Academic Press, New York, pp. 146-152.

Kitaysky, A.S., Kitaiskaia, E.V., Piatt, J.F., Wingfield, J.C., 2003. Benefits and costs of increased levels of corticosterone in seabird chicks. Hormones and Behavior 43, 140-149.

Kitaysky, A.S., Kitaiskaia, E.V., Wingfield, J.C., Piatt, J.F., 2001a. Dietary restriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. Journal of Comparative Physiology - B, Biochemical, Systemic, and Environmental Physiology 171, 701-709.

Kitaysky, A.S., Piatt, J.F., Wingfield, J.C., Romano, M., 1999. The adrenocortical stress-response of Black-legged Kittiwake chicks in relation to dietary restrictions. Journal of Comparative Physiology - B, Biochemical, Systemic, and Environmental Physiology 169, 303-310.

Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 2001b. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. Behavioral Ecology 12, 619-625.

Lin, H., Sui, S.J., Jiao, H.C., Buyse, J., Decuypere, E., 2006. Impaired development of broiler chickens by stress mimicked by corticosterone exposure. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 143, 400-405.

Lindén, M., Gustafsson, L., Pärt, T., 1992. Selection on Fledging Mass in the Collared Flycatcher and the Great Tit. Ecology 73, 336-343.

Lindström, J., 1999. Early development and fitness in birds and mammals. Trends in Ecology and Evolution 14, 343-348.

Loiseau, C., Sorci, G., Dano, S., Chastel, O., 2008. Effects of experimental increase of corticosterone levels on begging behavior, immunity and parental provisioning rate in house sparrows. General and Comparative Endocrinology 155, 101-108.

Love, O.P., Bird, D.M., Shutt, L.J., 2003. Corticosterone levels during post-natal development in captive American kestrels (*Falco sparverius*). General and Comparative Endocrinology 130, 135-141.

McNabb, F.M.A., Scanes, C.G., Zeman, M., 1998. Endocrine Control of Development. In: Starck, J.M., Ricklefs, R.E. (Eds.), Avian Growth and Development. Oxford University Press, New York, pp. 174-202.

Metcalfe, N.B., Monaghan, P., 2001. Compensation for a bad start: grow now, pay later? Trends in Ecology and Evolution 16, 254-260.

Moe, B., Brunvoll, S., Mork, D., Brobakk, T.E., Bech, C., 2004. Developmental plasticity of physiology and morphology in diet-restricted European shag nestlings (*Phalacrocorax aristotelis*). Journal of Experimental Biology 207, 4067-4076.

Müller, C., Almasi, B., Roulin, A., Breuner, C.W., Jenni-Eiermann, S., Jenni, L., 2009. Effects of corticosterone pellets on baseline and stress-induced corticosterone and corticosteroid-binding-globulin. General and Comparative Endocrinology 160, 59-66.

Naef-Daenzer, B., Widmer, F., Nuber, M., 2001. Differential post-fledging survival of great and coal tits in relation to their condition and fledging date. Journal of Animal Ecology 70, 730-738.

Negro, J.J., Chastin, A., Bird, D.M., 1994. Effects of short-term food deprivation on growth of hand-reared American kestrels. Condor 96, 749-760.

Oyan, H.S., Anker-Nilssen, T., 1996. Allocation of growth an food stressed Atlantic puffin chicks. Auk 113, 830-841.

Payne, R.W., 2003. The Guide to GenStat Release 7.1., Part 2: Statistics. VSN International, Oxford.

Post, J., Rebel, J.M.J., ter Huurne, A.A.H.M., 2003. Physiological Effects of Elevated Plasma Corticosterone Concentrations in Broiler Chickens. An Alternative Means by Which to Assess the Physiological Effects of Stress. Poultry Science 82, 1313-1318.

Pravosudov, V.V., Kitaysky, A.S., 2006. Effects of nutritional restrictions during post-hatching development on adrenocortical function in western scrub-jays (*Aphelocoma californica*). General and Comparative Endocrinology 145, 25-31.

Richner, H., 1989. Habitat-Specific Growth and Fitness in Carrion Crows (*Corvus corone corone*). Journal of Animal Ecology 58, 427-440.

Romero, L.M., Strochlic, D., Wingfield, J.C., 2005. Corticosterone inhibits feather growth: Potential mechanism explaining seasonal down regulation of corticosterone during molt. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 142, 65-73.

Saino, N., Suffritti, C., Martinelli, R., Rubolini, D., Moller, A.P., 2003. Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (*Hirundo rustica*). Behavioral Ecology 14, 318-325.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21, 55-89.

Schew, W.A., Ricklefs, R.E., 1998. Developmental Plasticity. In: Starck, J.M., Ricklefs, R.E. (Eds.), Avian Growth and Development. Oxford University Press, New York, pp. 288-304.

Schew, W.A. The evolutionary significance of developmental plasticity in growing birds. 1995. Dissertation, University of Pennsylvania.

Searcy, W.A., Peters, S., Nowicki, S., 2004. Effects of early nutrition on growth rate and adult size in song sparrows *Melospiza melodia*. Journal of Avian Biology 35, 269-279.

Siegel, P.B., Gross, W.B., Dunnington, E.A., 1989. Effects of dietary corticosterone in young Leghorn and Meat-type cockerels. British Poultry Science 30, 185-192.

Sokal, R.R., Rohlf, F.J., 2000. Biometry. W. H. Freeman and company, New York.

Stearns, S., Hoekstra, R., 2005. Evolution. Oxford University Press, Oxford.

Strochlic, D.E., Romero, L.M., 2008. The effects of chronic psychological and physical stress on feather replacement in European starlings (*Sturnus vulgaris*). Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 149, 68-79.

Thompson, R., Welham, S.J., 2003. REML analysis of mixed models. In: Payne, R.W. (Ed.), The Guide to GenStat Release 7.1., Part 2: Statistics VSN International, Oxford, pp. 513-607.

Wada, H., Breuner, C.W., 2008. Transient elevation of corticosterone alters begging behavior and growth of white-crowned sparrow nestlings. Journal of Experimental Biology 211, 1696-1703.

Wingfield, J.C., Maney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M., Richardson, R.D., 1998. Ecological Bases of Hormone-Behavior Interactions: The "Emergency Life History Stage". American Zoologist 38, 191-206.

Chapter 3

Development of the adrenocortical response to stress in Eurasian kestrel nestlings *Falco tinnunculus*: the importance of condition and brood hierarchy

Claudia Müller, Susanne Jenni-Eiermann and Lukas Jenni

Abstract

The developmental hypothesis proposes that the adrenocortical response to stress during postnatal development in birds should not develop when the benefits of elevated corticosterone do not outweigh the deleterious effects on growth and development. We investigated three predictions developed from the developmental hypothesis in free-living Eurasian kestrel *Falco tinnunculus* nestlings, a semi-altricial species with partial hatching asynchrony. We measured baseline and handling-induced corticosterone levels, CBG capacity and free corticosterone levels on day 10 and 21 of age and related these to brood hierarchy, the development of the defence behaviour and body condition (fat stores). The first prediction, namely that the adrenocortical response to handling and human presence at the nest should increase during the time when nestlings developed the ability to defend themselves, was confirmed. Although already relatively high on day 10, the adrenocortical response to handling was higher on day 21 when nestlings defended themselves vigorously and reacted to our presence at the nest. We also confirmed the second prediction, namely that only nestlings with adequate energy stores should mount an adrenocortical stress response to an acute stressor, while nestlings with low energy stores should avoid additional energy expenses. While baseline corticosterone levels were negatively related to body energy stores, increase in corticosterone to handling was positively related. Confirming our third prediction, we showed that in free-living kestrels both baseline corticosterone levels and the adrenocortical response to handling were not related to hatching order *per se*, but were predominantly determined by body condition. This is in contrast to studies with ad libitum fed nestlings in the lab, which may show an underlying pattern of decreasing corticosterone levels with hatching order which, in free-living birds under field conditions, is superimposed by variation in body condition. Since CBG capacity did not vary with any of the parameters investigated, free corticosterone levels showed a similar pattern as total levels. We argue that the variation of the postnatal adrenocortical response to stress is an adaptive modulation which is particularly important as elevated corticosterone may adversely affect the phenotype for life.

1. Introduction

Adults of all vertebrates counteract adverse effects on their homeostasis by a hypothalamic-pituitary-adrenal (HPA) stress response that results in an increased secretion of glucocorticoids (corticosterone in birds, e.g. Sapolsky et al., 2000). However, an adrenocortical stress response also entails costs. These may be short-term, such as an increased energy expenditure (DuRant et al., 2008), or long-term; during growth elevated corticosterone levels adversely affect growth and development and may profoundly change the phenotype (e.g. Kitaysky et al., 2003; Müller et al., 2009b). Life-history theory predicts that an adrenocortical response to stress, eliciting behavioural and physiological responses to overcome the stressful situation, should only arise, if the animal has the potential to ameliorate its situation, i.e. if the benefits outweigh the costs.

Altricial nestlings do not have the ability to move away from the nest to escape from a predator, avoid environmental extremes or search for food by themselves. Thus, the recently published developmental hypothesis (e.g. Sims and Holberton, 2000; Blas et al., 2006) states, that during postnatal development the adrenocortical response to stress should only develop, when the benefits of elevated corticosterone outweigh the costs. The developmental hypothesis is supported at the inter-specific level by studies showing that altricial chicks do not show an adrenocortical response to stress after hatching despite a fully functional adrenocortical tissue (Sims and Holberton, 2000), while precocial chicks do (Holmes et al., 1989). At the intra-specific level, the developmental hypothesis predicts a gradual increase of the adrenocortical stress response with age in altricial and semi-altricial nestlings, i.e. with the developing physiological and behavioural abilities of the nestlings. Such a postnatal increase of the adrenocortical response to stress has been described in five altricial and semi-altricial species (Sims and Holberton, 2000; Love et al., 2003a; Walker et al., 2005; Blas et al., 2006; Wada et al., 2007), while in a precocial species it was strong from the first day of hatching and decreased during postnatal development (Holmes et al., 1989).

Thus there is general support for the developmental hypothesis at the inter-specific level and to some degree at the intra-specific level (changes of adrenocortical stress response with age). However, at the intra-specific level the general predictions remain largely untested. In this study, we examined three predictions, which are developed in the following.

To date, the gradual development of the stress response in altricial and semi-altricial species was explained by the gradual development of behavioural and physiological abilities in general, but an investigation on relationships between behaviour and stress response is lacking. Potential functions of elevated corticosterone during postnatal development of an altricial nestling include behavioural changes to improve nutritional input, such as an increase in begging to receive more food from parents (Kitaysky *et al.*, 2003) or more dominant (or submissive) behaviour in competition with siblings (e.g. Ramos-Fernandez et al., 2000). Threats to nestlings also include predator attacks. Some species, e.g. the Falconiformes, have developed a pronounced defence behaviour against nest intruders. Because this situation is similar to a predator attack in adult birds

resulting in a fight or flight response (e.g. Sapolsky *et al.*, 2000), we predict (1) a higher adrenocortical stress response in those nestlings able to defend themselves against nest predators. It follows that the adrenocortical stress response should increase with the ability of defence, and that nestlings able to defend themselves should react to the presence of humans at the nest by increasing corticosterone, while nestlings unable to defend themselves should not.

An adrenocortical response to a stressor is costly, because it increases energy expenditure (DuRant *et al.*, 2008) and mobilizes the internal fat and protein stores, similar as in adults (e.g. Sapolsky *et al.*, 2000). Therefore, we predict (2) that only nestlings with adequate energy stores should mount an adrenocortical stress response to an acute stressor, while nestlings with low energy stores should avoid additional energy expenses. To date, there is mixed evidence on the effect of body energy stores on the adrenocortical response to handling in nestlings (Kitaysky et al., 1999; Blas et al., 2005; Love and Williams, 2008) and juveniles (Heath and Dufty, 1998). Similar as in adults (e.g. Jenni et al., 2000), nestling baseline corticosterone levels are often negatively correlated with fat reserves or body condition, especially in free-living birds (Sockman and Schwabl, 2001) or in food-restriction experiments (e.g. Kitaysky *et al.*, 1999). In ad libitum food studies however the opposite pattern or no pattern was found (e.g. Love et al., 2003b).

Asynchronous hatching entails a hierarchy within the brood. In captive, *ad libitum* fed broods, a relationship between rank and baseline (Schwabl, 1999; Love *et al.*, 2003b) and handling-induced (Love *et al.*, 2003b) corticosterone levels was found, with first-hatched nestlings having higher levels of both baseline and handling-induced corticosterone than younger siblings. In American kestrels *Falco sparverius* this pattern was modified in that a greater hatching span between siblings increased the effects of rank (Love *et al.*, 2003b). These authors suggested that hierarchies in adrenocortical function among siblings may maintain developmental hierarchies in that the older nestlings through higher corticosterone levels beg more and receive more food; however, begging rates have not been measured, food was not a limiting factor under the lab conditions, and corticosterone levels have not been measured on the same day within the brood. Studies in free-living birds have found the opposite pattern (younger nestlings having higher baseline corticosterone levels; Eraud et al., 2008) or no dependence of baseline and stress-induced corticosterone levels on nestling rank (Sockman and Schwabl, 2001; Blas et al., 2005). From the developmental hypothesis and the prediction developed above, we suggest another explanation and predict that (3) the adrenocortical stress response predominantly depends on body energy stores, rather than hatching order and, thus older nestlings may have a stronger adrenocortical stress response if they have more body energy stores than younger siblings within a brood. It also follows that younger siblings reach similar or higher baseline corticosterone levels than older siblings if their body condition is poor.

The adrenocortical stress response may be modulated not only by variation in the release of corticosterone, but also by variation of the binding capacity of corticosterone binding globulins CBG. CBG capacity determines the concentration of circulating free corticosterone, the fraction

considered to be biologically active (Breuner and Orchinik, 2002). We therefore not only measured total circulating corticosterone, but also the binding capacity of corticosterone binding globulins CBG, and thus were able to estimate free corticosterone levels.

In this study, we examined the three predictions developed above in broods of the Eurasian kestrel *Falco tinnunculus*, a semi-altricial species with partial hatching asynchrony. Because we examined free-living broods in the natural context, we had chicks with different body conditions and thus could examine in natural conditions how body condition affects the development of the adrenocortical response to stress.

2. Material and Methods

2.1. Study species and study sites

The Eurasian kestrel *Falco tinnunculus* is a small raptor species with reversed size-dimorphism. It breeds in open landscapes and hunts on small vertebrates (especially voles) and large insects. The kestrel female incubates the 3 - 7 eggs for 29 days, generally beginning after the third egg. Therefore, the three oldest nestlings within a brood usually have the same age. The nestlings stay in the nest for 32 to 39 days.

The field work was performed in the Jura mountains and river valleys in North-western Switzerland (7°60'E / 47°30'N), where Eurasian kestrels breed in nest boxes mounted on agricultural buildings in open rural landscapes. In 2004 and 2005, 285 nestlings of 66 broods within an area of approximately 600 km^2 were investigated.

2.2. Breeding parameters

From April onwards, nest boxes were checked biweekly for clutches and, before hatching, every second day to determine the hatching date of the oldest nestlings. Later-hatched nestlings were aged by a combination of these regular controls and the wing length on day 10. For each nestling, we calculated the age difference to the oldest siblings within the brood (in days, 0d: 118 nestlings, -1 day: 98 nestlings; -2 days: 43 nestlings, -3 days: 13 nestlings, -4 days: 11 nestlings, -5 days: 2 nestlings). For each brood, we determined the hatching span (in days), i.e. the age difference between the oldest and the youngest nestling. Brood size ranged from 1 to 7 nestlings.

2.3. Blood sampling

Blood samples were taken when the oldest nestlings were 10 and 21 days old (parameter: *age*). For logistical reasons, not all broods could be sampled on the intended age and the age of the old-

est nestlings ranged from 9 to 11 (54 of 66 broods sampled at 10 days) and from 20 to 23 days (51 of 65 broods sampled at 21 days), respectively (parameter: *brood age*).

For blood sampling, a first batch of 2 - 3 nestlings was removed from the nest box and a blood sample was taken from the alar vein within 3 minutes (mean ± SD = 1.88 ± 0.62 min, n = 398). 17 min (mean 16.60 ± 2.35 min, range 14 – 21 min) after capture, a second, handling-induced blood sample was taken at the other wing. When returning this first batch of nestlings to the nest box, the second batch of the brood was removed from the nest box and blood sampled and measured. In a few large broods, nestlings were divided in three batches. A possible adrenocortical response of the second and third batch to our presence at the nest and the capture of siblings was assessed by introducing the batch number into the analysis.

After the first blood sample, nestlings were measured and then held in a cloth bag until the second blood sample. Unfortunately, we have no data about the time course of the adrenocortical response to handling, but considering the course of corticosterone with time after capture in nestlings of the closely related American kestrel (Love *et al.*, 2003a), our handling-induced corticosterone levels 17 min after capture seem to be near the maximum levels reached.

Blood was collected with heparinized capillary tubes (80 μl blood per sample), immediately transferred to Eppendorf tubes and centrifuged within 1 hour. Plasma and blood cells were separated and stored in liquid nitrogen until transfer to the lab, where they were stored at –20°C until analysis. Plasma was used for corticosterone and CBG analysis, blood cells served for sex determination. Blood sampling was done under license of the animal experiment permit n°274 from the Cantonal Veterinarian Office of Baselland.

2.4. Body condition measurements and sex determination

As in passerines (Kaiser, 1993), we assessed the amount of subcutaneous fat stores between the furcula by assigning a fat score ranging from 0 to 4 (0: no visible fat; 1: 1 mm stripe of fat at the bottom of the furcular pit; 2: fat stripes 2-3 mm broad; 3: furcular pit nearly covered with fat (about 75%), 4: furcular pit completely filled with fat).

Nestlings were sexed with molecular methods by fragment analysis on CHD1W/CHD1Z (Fridolfsson and Ellegren, 1999) using blood cells extracted with the QIAamp DNA extraction Kit (Qiagen) in 2004 and after Kawasaki (1990) in 2005. Samples of 2004 were analysed at the Swiss Federal Institute for Forest, Snow and Landscape Research in Birmensdorf, Switzerland and those of 2005 at the Agroscope Research Station ACW in Wädenswil, Switzerland.

2.5. Corticosterone assay

Plasma corticosterone concentration was determined using an enzyme immuno assay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988). Corticosterone in 5 μl plasma and 195 μl water was

extracted with 4 ml dichlormethane, re-dissolved in phosphate buffer and given in triplicates in the enzyme immuno assay. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, Progesterone 0.004%, 18-OH-DOC 0.01%, Cortisol 0.12%, 18-OH-B 0.02% and Aldosterone 0.06%) was 1:8'000. HRP (1:400'000) linked to corticosterone served as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. Plasma pools from chickens with two different corticosterone concentrations were included as internal controls on each plate. If the concentration was below the detection threshold, the determination was repeated with 10 µl plasma. If the concentration was still below the detection threshold, the value of the lowest detectable concentration (1 ng ml^{-1}) was assigned (23 samples). Intra-assay variation ranged from 4.5 to 10.8 % and inter-assay variation from 9.6 to 17.6 %, depending on the concentration of the internal control and the year of determination.

2.6. Corticosteroid-binding-globulin

The affinity and capacity of corticosteroid-binding-globulin (CBG) was measured with a radioligand-binding assay with tritiated corticosterone following Breuner et al. (2003). For point sample analysis, 10-15 µl plasma were stripped of endogenous steroids with 2 parts of dextran-coated charcoal (0.1% dextran, 1% Norit A charcoal in 50 mM Tris) for 30 minutes at room temperature. Outside this stripping procedure, the plasma was maintained below 4°C. The final assay dilution of plasma samples was 1:99. The binding assay was carried out in 50 nM Tris buffer at 4°C and terminated after 2 hours. 1 hour before filtering, glass fiber filters (Whatman) were soaked in 25 nM Tris with 0.3% polyethylenimine. After filtration, filters were rapidly rinsed with 3 rinses of 3 ml ice-cold 25 nM Tris. Point sample analysis was performed with individual plasma samples, for the saturation analysis pooled samples were run. For the saturation analyses, 0.25-12 nM [^{3}H]Corticosterone were incubated with pooled plasma with and without 1µM unlabeled corticosterone. 20 nM [^{3}H]Corticosterone was employed to estimate CBG capacity in individual birds. Affinity estimates obtained from equilibrium saturation analysis (Kd = 4.59, see Müller et al., 2009a) indicated that this ligand concentration occupies ~80% of total binding sites. For analysis, the samples were corrected to 100% capacity within each assay. The intra-assay variation was 7.1 %, the inter-assay variation 7.70 %.

The equation of Barsano and Baumann (1989) was used to estimate free corticosterone titers from total corticosterone concentrations and CBG binding parameters

$$H_{free} = 0.5 \times \left[H_{total} - B_{max} - 1/K_a\right] \pm \sqrt{(B_{max} - H_{total} + 1/K_a)^2 + 4(H_{total}/K_a)}$$

where H_{free} is free hormone, H_{total} is total hormone, B_{max} is total binding capacity of CBG, and K_a = 1/dissociation constant (K_d) (all values in nM). CBG capacity was measured in all baseline samples.

2.7. Data analysis

The effect of age on corticosterone levels, CBG capacity and furcular fat score was assessed in a repeated measures mixed model, including the age (factor, day 10 versus day 21) as fixed and nestling nested within brood as random factors. The dependence of baseline and handling induced total and free corticosterone levels and CBG capacity on batch number (factor, 3 levels), brood age (covariate), brood size (covariate), age difference to the oldest sibling within the brood (covariate), hatching span (covariate), sex (factor), furcular fat score (factor, 5 levels) and the interactions age difference to the oldest sibling x sex and age difference to the oldest sibling x hatching span were analysed in separate mixed models with brood introduced as random factor (Type I, parameters sequentially introduced into the model). These interactions were introduced to examine whether later-hatched males or females had different corticosterone levels (size dimorphism) and whether a large hatching span interacted with corticosterone levels, similar as in Love et al. (2003b). Baseline total and free corticosterone and CBG capacity were square root-transformed to obtain normality of residuals. To investigate whether younger nestlings within the brood had smaller subcutaneous fat stores than older siblings, we tested in different mixed models for day 10 and 21 separately, whether furcular fat score depended on the age difference to the oldest sibling (introduced as fixed covariate). As above, brood identity was introduced as random term, to correct for non-independence of siblings. All mixed model analyses were performed in Genstat 9 (Payne, 2003; Thompson and Welham, 2003).

To assess whether the nestlings within a brood, ordered according to their corticosterone levels on day 10, kept their corticosterone rank on day 21, we calculated Spearman's rank correlations (with SPSS 12.0). Broods with only one nestling or more than one third of nestlings with missing values were excluded from these analyses.

Corticosterone levels began already to rise slightly within 3 min after capture ($F = 4.183$, $d.f. = 1$, $p = 0.041$, $n = 401$), but the increase between 2 and 3 minutes after capture was only 1 ng/ml total corticosterone and we did not consider this slight increase in order to enlarge our sample size. Corticosterone rose more strongly after 3 min after capture and, therefore, blood samples taken after 3 min after capture were excluded from the analysis. Because it was more difficult to capture 21 days old nestlings than 10 d old ones, 30% of these samples were taken after 3 min and excluded from the analysis. On day 10 one nestling, hatched 5 d after the oldest sibling, had baseline and handling-induced total corticosterone levels of 103 and 100 ng/ml and it starved between day 10 and 13. Because this outlier had a strong levering effect, it was excluded from all analyses.

3. Results

3.1. Postnatal development of the adrenocortical response to handling

While there was no difference in baseline total corticosterone between day 10 and 21 (repeated measures mixed model; mean ± SE 6.71 ± 0.47 ng/ml versus 7.08 ± 0.43 ng/ml , Fig. 1, Wald = 2.07, d.f. = 1, p = 0.150), CBG capacity increased from 61.36 ± 2.10 nmol on day 10 to 77.23 ± 3.10 nmol on day 21 (Wald = 25.99, d.f. = 1, p < 0.001). However, baseline free corticosterone levels did not differ significantly between day 10 and 21 (1.26 ± 0.21 ng/ml versus 0.98 ± 0.16, Wald = 1.32, d.f. = 1, p = 0.250).

Handling-induced total corticosterone levels 17 min after capture increased from 20.26 ± 0.59 on day 10 to 24.54 ± 0.71 ng/ml on day 21 (Fig. 1; Wald = 21.74, d.f. = 1, p < 0.001). The increase in total corticosterone as a response to handling (difference between baseline and handling-induced total corticosterone) also became stronger with age (increase of 13.71 ± 0.58 ng/ml on day 10, 16.98 ng/ml on day 21; Fig. 1; Wald = 18.58, d.f. = 1, p < 0.001).

While there was no effect of brood age on handling-induced corticosterone around day 10 (Table 1), handling-induced corticosterone levels around day 21 increased significantly with brood age over only 4 days (mixed model; Table 2, Fig. 2).

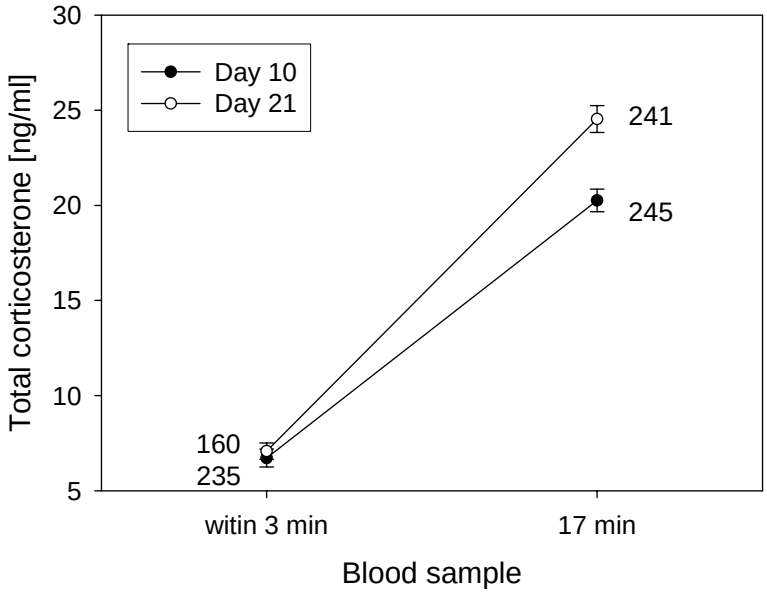

Fig. 1. Mean (± SE) baseline (within 3 minutes) and handling-induced (17 min after capture) total corticosterone levels of free-living kestrel nestlings on nestling day 10 and 21. Nestlings of 61 broods were sampled on day 10 and day 21, numbers near error bars indicate sample sizes. Because not all nestlings could be sampled within 3 min, sample sizes are lower than at 17 min.

Table 1. Dependence of baseline total and free corticosterone, CBG capacity, handling-induced total corticosterone and increase in total corticosterone, each as dependent variable in a separate mixed model, on various explanatory variables (fixed factors) at the age of 10 days. Brood identity was introduced as random factor.

Day 10		Baseline						Handling-induced			
		Total cort (n=221)		Free cort (n=198)		CBG cap. (n=198)		Total cort (n=232)		Increase in cort (n=197)	
Explanatory variable	d.f.	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p
Batch (capture group at nest)	2	3.84	0.147	2.27	0.321	0.19	0.911	-	-	-	-
Brood age [d]	1	0.30	0.586	0.57	0.451	0.01	0.932	0.12	0.726	0.50	0.479
Brood size	1	0.04	0.846	0.05	0.816	2.88	0.090	0.53	0.465	2.17	0.141
Age difference to the oldest sibling[d]	1	3.26	0.071	5.65	0.018	3.38	0.066	0.05	0.816	1.37	0.242
Hatching span [d]	1	0.03	0.855	1.16	0.282	0.04	0.845	0.05	0.816	0.04	0.846
Sex	1	0.38	0.536	0.17	0.680	0.01	0.907	0.77	0.379	2.09	0.149
Furcula fat score	2	18.74	<0.001	19.11	<0.001	0.09	0.957	7.95	0.019	8.64	0.013
Age difference to oldest sibling x Sex	1	0.73	0.394	0.36	0.549	0.49	0.483	0.42	0.515	0.16	0.689
Age difference to oldest sibling x Hatching span	1	0.79	0.374	0.66	0.416	0.02	0.875	0.02	0.883	0.39	0.532

Table 2. Dependence of baseline total and free corticosterone, CBG capacity, handling-induced total corticosterone and increase in total corticosterone, each as dependent variable in a separate mixed model, on various explanatory variables (fixed factors) at the age of 21 days. Brood identity was introduced as random factor.

Day 21		Baseline						Handling-induced			
		Total cort (n=150)		Free cort (n=144)		CBG cap. (n=145)		Total cort (n=230)		Increase in cort (n=149)	
Explanatory variable	d.f.	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p
Batch (capture group at nest)	2	19.04	<0.001	6.81	0.033	5.97	0.050	-	-	-	-
Brood age [d]	1	1.89	0.169	0.46	0.499	2.58	0.108	6.50	0.011	1.80	0.179
Brood size	1	1.16	0.282	0.34	0.561	0.07	0.795	0.06	0.805	0.03	0.874
Age difference to the oldest sibling [d]	1	2.83	0.093	1.96	0.162	0.85	0.357	0.26	0.612	0.14	0.711
Hatching span [d]	1	0.14	0.712	0.07	0.791	1.55	0.213	0.01	0.915	0.29	0.593
Sex	1	1.18	0.278	0.49	0.484	0.21	0.644	2.87	0.090	3.36	0.067
Furcula fat score	3	7.51	0.057	5.66	0.129	1.40	0.705	2.95	0.399	3.07	0.381
Age difference to oldest sibling x Sex	1	0.51	0.475	0.85	0.357	0.58	0.445	1.88	0.170	0.26	0.607
Age difference to oldest sibling x Hatching span	1	4.72	0.030	2.29	0.130	0.01	0.920	0.45	0.502	0.18	0.676

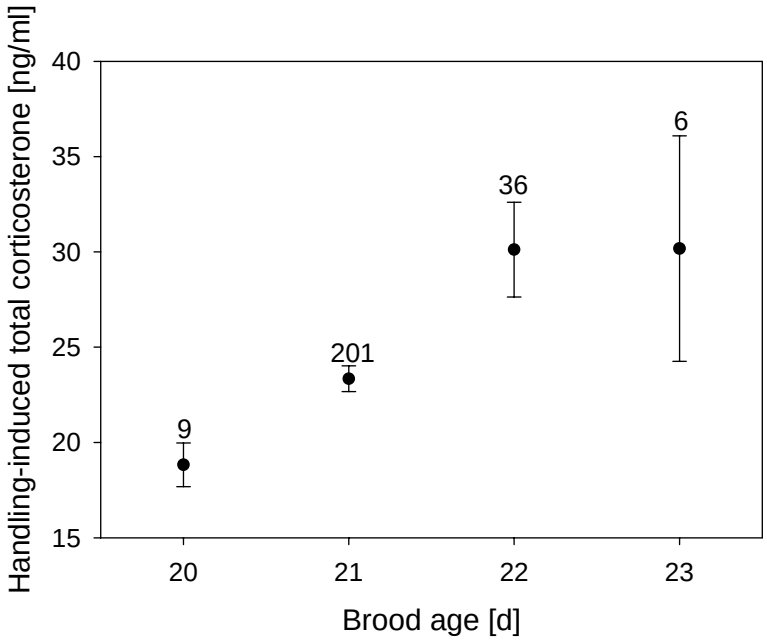

Fig. 2. Dependence of handling induced (17 min after capture) total corticosterone levels on brood age (age of the oldest sibling within the brood) around day 21. Free-living kestrel nestlings of 61 broods were sampled at one of the brood ages indicated. Numbers near error bars (mean ± SE) indicate sample sizes.

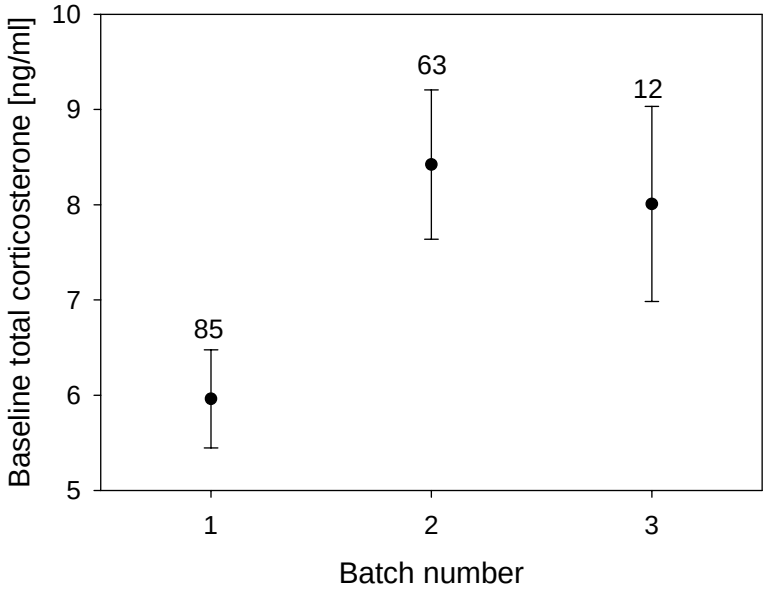

Fig. 3. Mean baseline total corticosterone levels (± SE) of free-living kestrel nestlings on day 21 plotted against batch number. Nestlings of batch 1 were captured immediately after arrival at the nest box, nestlings of batch 2 22.0 ± 0.61 (mean ± SD) min after arrival and nestlings of batch 3 40.9 ± 2.0 min after arrival. Numbers near error bars indicate sample sizes.

3.2. Behavioural and adrenocortical response to human presence at the nest

When visiting the nest at the age of 10 days, nestlings normally were arranged to a heat pyramid to minimize temperature loss and were relatively passive when taking them out of the nest. They did not show any anti-predator-behaviour during our quiet presence at the nest. In contrast, at the age of 21 days, nestlings were spread out in the nest box and showed an extensive defence behaviour towards us by lying on their back and repulsing with their talons when being captured, continuously emitting alarm calls.

Baseline total corticosterone on day 10 was similar in all batches of nestlings, indicating no adrenocortical response to our presence at the nest when capturing the siblings. However, on day 21, nestlings of batch 2 and 3 showed significantly higher baseline total corticosterone levels than nestlings of batch 1 (mixed model; Table 2; Fig. 3).

.

3.3. Effects of body condition, hatching order and brood size

On day 10, the furcular fat score ranged from 0 to 2 (mean ± SE: 1.13 ± 0.04 scores). Younger nestlings within a brood had less furcular fat stores than older nestlings (mixed model, Wald = 50.26, d.f. = 1, $p < 0.001$, Fig. 4). Nestlings with a fat score of 0 had significantly higher total baseline corticosterone levels (mixed model; Table 1, Fig. 5a) than nestlings with a score of 1 or 2 and this was also found for free baseline corticosterone (not shown).

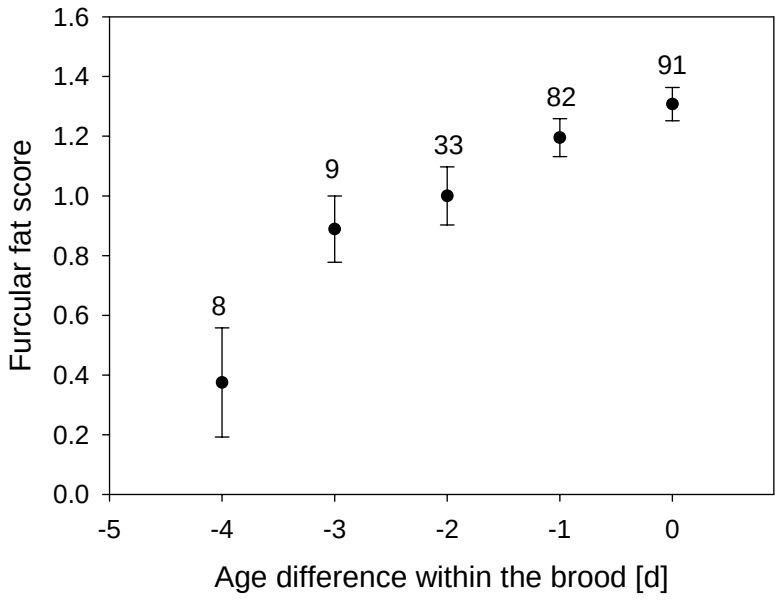

Fig. 4. Mean furcular fat score (± SE) when the oldest nestlings were 10 days old, depending on the age difference to the oldest nestling within the brood. Numbers near error bars indicate sample sizes.

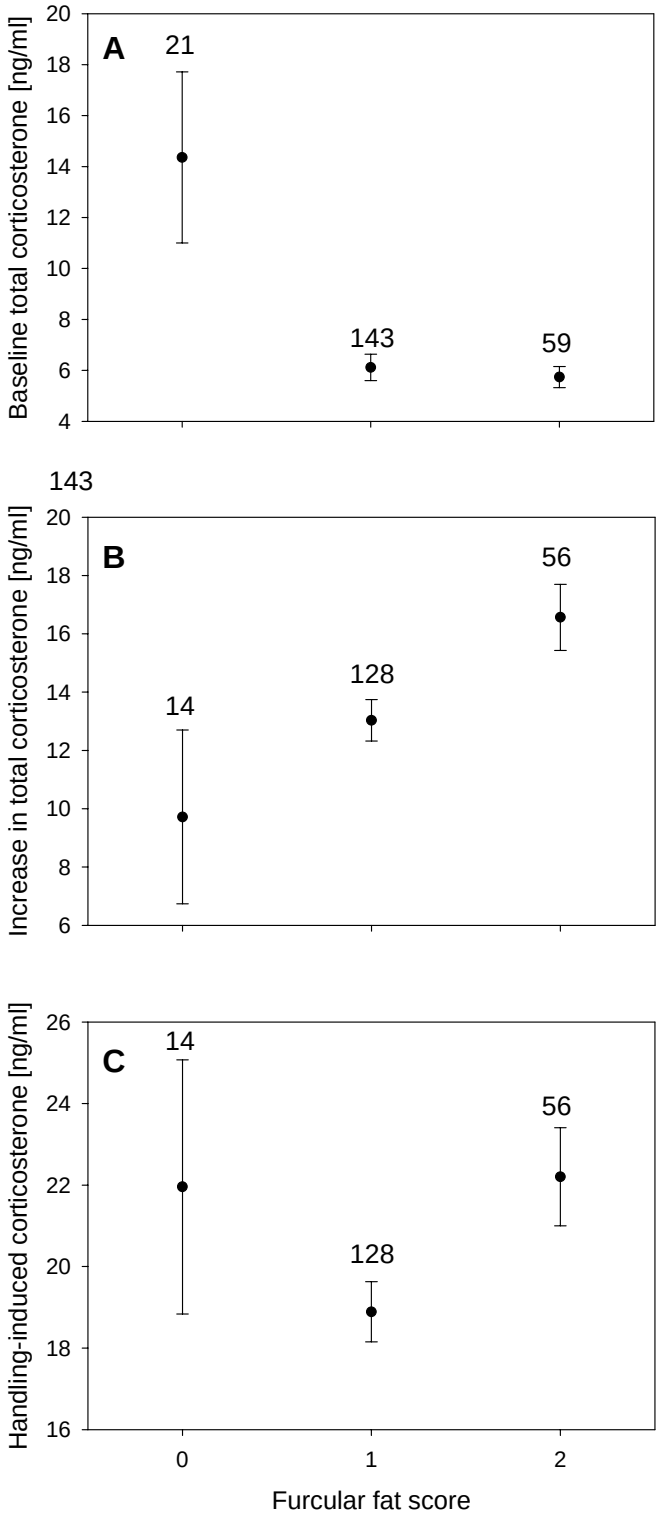

Fig. 5. Mean (± SE) baseline total corticosterone (A), increase in total corticosterone from within 3 to 17 min after capture (B) and handling-induced total corticosterone level (17 min after capture) (C) of free-living kestrel nestlings, plotted against furcular fat score on day 10. Numbers near error bars indicate sample sizes.

For handling-induced corticosterone levels, the relationship with fat stores was the opposite. Nestlings with a fat score of 2 showed a significantly stronger increase in total corticosterone levels and higher handling-induced corticosterone levels than those with a fat score of 0 or 1 (Table 1, Fig. 5b, 5c). On day 21, furcular fat score was significantly higher than on day 10 (repeated measures mixed model, Wald = 549.45, d.f. = 1, $p < 0.001$), ranged from 1 to 4 (2.58 ± 0.05 scores) and showed no significant relation with corticosterone levels (Wald = 2.85, d.f. = 1, $p = 0.091$).

Baseline and handling-induced corticosterone levels and CBG capacity on day 10 and 21 did not depend on brood size (Table 1 and 2). On day 10, younger nestlings within a brood had higher baseline free corticosterone levels than older nestlings, and baseline total corticosterone

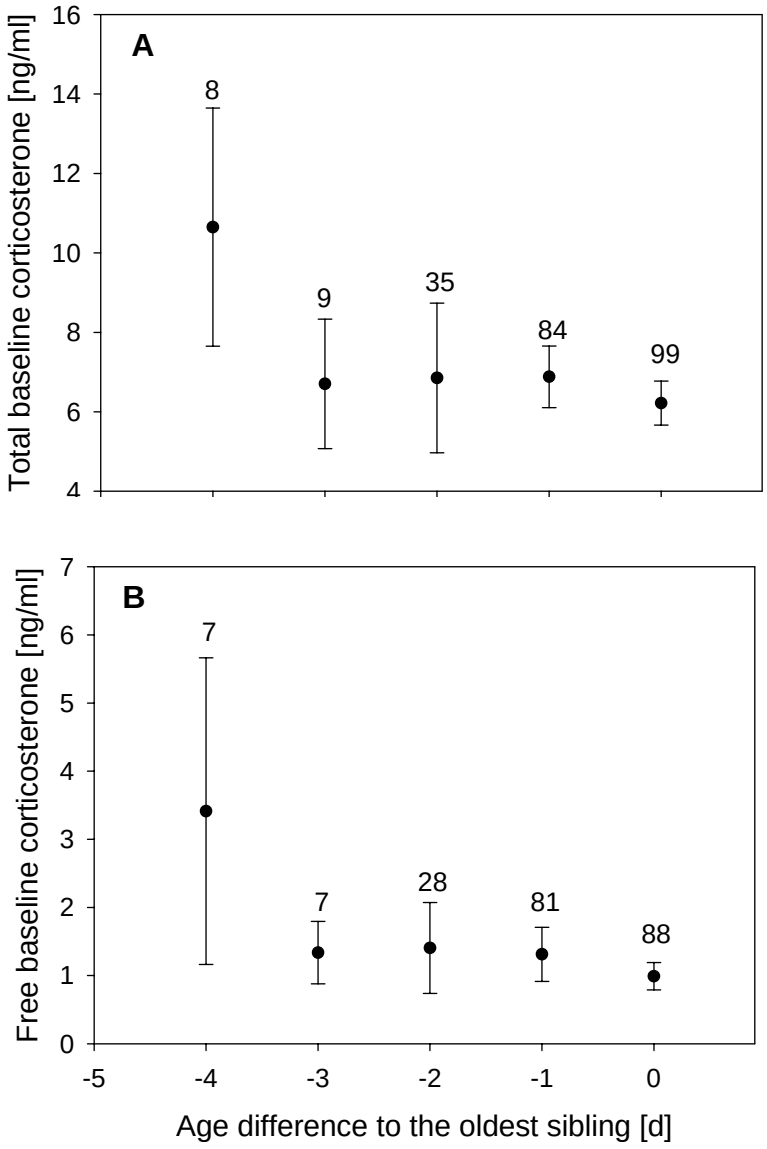

Fig. 6. Mean (± SE) baseline total (A) and free (B) corticosterone levels of kestrel nestlings on day 10, plotted against the age difference to the oldest sibling within the brood. Numbers near error bars indicate sample sizes.

levels showed the same non-significant tendency (Table 1, Fig. 6). This relationship seemed to depend predominantly on the worse condition of younger nestlings within the brood. When introducing the furcular fat score before the age difference to the oldest sibling into the model, the age difference was not significant anymore (mixed model, Wald = 0.09, d.f. =1, p = 0.763), but fat score was (Wald = 21.93, d.f. = 2, p < 0.001). On day 21, the later-hatched nestlings in broods with a large hatching span had even higher baseline total corticosterone levels than later-hatched nestlings of broods with a smaller hatching span (interaction age difference to the oldest sibling x hatching span significant, Table 2).

We found no significant differences in any corticosterone measures or CBG capacity between the sexes (Tables 1 and 2), although male nestlings showed a tendency to have lower handling-induced total corticosterone levels than females on day 21 (Table 2, mean ± SE: m = 23.07 ± 1.04 ng/ml, f = 25.92 ± 0.97 ng/ml).

The rank of the nestlings within the brood according to their total baseline corticosterone values on day 10 was correlated with the corresponding rank on day 21 (Spearman's rho = 0.145, n = 227, p = 0.024), and a similar correlation was found for total handling-induced corticosterone (Spearman's rho = 0.166, n = 184, p = 0.025). However, the exact baseline corticosterone rank within the brood was kept from day 10 to 21 by only 26.4% of the nestlings, the exact handling-induced corticosterone rank by 34.2% of the nestlings.

4. Discussion

4.1. Postnatal development of the adrenocortical response to a threat

In this study we showed that the adrenocortical response to handling as well as to human presence at the nest increased during the time when nestlings developed the ability to defend themselves. Thus our first prediction derived from the developmental hypothesis was confirmed, namely that the adrenocortical stress response increases with age, in parallel with the defence capabilities of the nestlings.

A similar increase in the adrenocortical response to handling with age was observed in other altricial and semi-altricial species (Sims and Holberton, 2000; Love et al., 2003a; Walker et al., 2005; Blas et al., 2006; Wada et al., 2007). However, while typical altricial passerine species have a very low adrenocortical response to handling early after hatching and a considerable increase with age (Sims and Holberton, 2000; Wada et al., 2007), the response to handling in the semi-altricial Eurasian kestrels (this study) and American kestrel (Love *et al.*, 2003a) was already considerable at the age of 10 days and showed only a small further increase to day 21. Possibly semi-altricial species which hatch at a more developed stage (with dunes, eyes open shortly after hatching) show an earlier development of the adrenocortical response to handling than true altricial species, thus may gain more, or have less disadvantages, from an activation of the HPA-axis.

As in the American kestrel (Love *et al.*, 2003a) Eurasian kestrels augment the adrenocortical response to handling around day 21 (Fig. 2). At this age, maximal body mass is normally reached and structural growth almost completed with the exception of feather growth. Hence, a strong rise in corticosterone levels can only have limited adverse effects on growth (contrary to possible serious long-term costs earlier in postnatal development, cf. Müller et al., 2009b) and therefore may be easily outweighed by possible benefits during the last phase in the nest (e.g. when nestlings are capable of defending themselves against predators) and the oncoming difficult and dangerous fledging phase (from day 32 onwards).

As predicted, the kestrel nestlings ready to defend themselves (day 21) also showed a slight increase in corticosterone levels as a response to the presence of humans at the nest, obviously perceived as a threat, while there was no such rise in corticosterone levels when nestlings were not able to defend themselves (day 10). We cannot distinguish, whether this adrenocortical response is triggered by our presence at the nest, or by the perception of the removal of their siblings, or the perception of the behavioural reaction (struggling and warning calls) of their supposedly threatened siblings. Black-legged kittiwake *Rissa tridactyla* nestlings also showed an age-dependent rise in corticosterone levels to the presence of a researcher removing their sibling from the nest (Fridinger et al., 2007).

One function of the adrenocortical response developing from day 10 to day 21 therefore seems to be to support a fight response to a predator attack. The rise in corticosterone levels as a response to a threat (our presence or handling) enables the allocation of additional energy to sustain the behavioural and physiological response and recover from it. Although nest-bound and dependent on their parents, older, altricial or semi-altricial nestlings, presumably especially raptors with potent tools (talons and beak), are able to beat back predators from the nest with this defence-and-fight response similar the “fight or flight”-response in adults (e.g. Sapolsky *et al.*, 2000).

In summary, it appears that the balance between costs and benefits of an adrenocortical response to a stressor changes with nestling age either because the benefits increase with age (e.g. developing self-defence) or because the costs decrease (e.g. less adverse effects on growth and development with age) or both. The lower adrenocortical response early in postnatal development is due to a lower corticosterone release and not by buffering total corticosterone with a higher CBG capacity resulting in lower free corticosterone, as found in white-crown sparrow *Zonotrichia leucophrys* nestlings (Wada *et al.*, 2007).

4.2. Dependence of the adrenocortical response on condition

We found that on day 10 the adrenocortical response to handling was positively related to subcutaneous fat stores (Fig. 5b). This confirms the second prediction that only nestlings with adequate energy stores can mount an adrenocortical stress response to an acute stressor, while nestlings

with low energy stores should avoid additional energy expenses. On day 21, nestlings generally have larger fat stores and this obviously allows a strong adrenocortical response in all nestlings.

In contrast to handling-induced levels, baseline corticosterone levels were high in birds without visible subcutaneous fat stores (Fig. 5a). A negative correlation between baseline corticosterone levels and body condition has also been found in food-restricted black-legged kittiwake nestlings (Kitaysky *et al.*, 1999) and free-living American kestrel nestlings (Sockman and Schwabl, 2001), similar as in many studies on adult birds (e.g. Müller et al., 2006; Jenni-Eiermann et al., 2008). Nestlings with no or little fat stores increase corticosterone levels to mobilize protein reserves for maintenance and growth (e.g. Carsia and Harvey, 2000). The absence of a negative relationship between baseline corticosterone levels and body condition in other studies can be explained by the fact that birds in bad condition were missing when fed ad libitum (e.g. in American kestrel nestlings, Love *et al.*, 2003b).

Because baseline corticosterone levels were high in birds without visible fat stores and approached handling-induced levels (Fig. 5a), the resulting handling-induced corticosterone levels were also high. Birds with little fat stores had low handling-induced corticosterone levels and birds with appreciable fat had high handling-induced corticosterone levels (Fig. 5c). Thus, through the combination of condition-dependent baseline corticosterone levels (Fig. 5a) and a handling-induced increase (Fig. 5b) resulted a non-linear dependence of handling-induced corticosterone levels on fat stores (Fig. 5c). This relationship was only apparent on day 10, but not on day 21. This is probably because body condition on day 10 was generally lower than on day 21. Food demand of kestrel nestlings increases around day 10, resulting in a large range of body conditions between siblings and subsequent brood reductions (own observation, Wiehn et al., 2000), while on day 21 body condition is generally better and most nestlings survive until fledging.

As in this study, a condition-dependent adrenocortical response to handling was found in American kestrel juveniles after fledging, with individuals in good condition showing a faster and steeper increase in corticosterone levels and subsequently a faster decrease to baseline levels than individuals in bad condition (Heath and Dufty, 1998). In contrast, other studies reported a negative correlation between handling-induced corticosterone levels and body condition (Kitaysky et al., 1999; Kitaysky et al., 2001; Love and Williams, 2008). This would correspond to the left leg of our u-shaped relationship (Fig. 5c). Indeed, these studies food-restricted the nestlings directly in the lab or via manipulation of the mothers and, therefore, might have missed nestlings with large body stores. This demonstrates that it is important to take into account body condition when interpreting corticosterone levels.

In summary, it appears that baseline and handling-induced corticosterone levels are highly condition-dependent. A growing bird in good condition seems to aim at having low baseline corticosterone levels and a potent (and short) adrenocortical response to an acute stressor. When body condition is poor, the adrenocortical response to an acute stressor is lower and when body condition is very poor, handling-induced levels are high because baseline levels have increased.

4.3. Corticosterone levels and hatching asynchrony

This study showed that in free-living Eurasian kestrels the adrenocortical response to handling was not related to hatching order (measured by age difference to the oldest nestlings), but only to body condition. The tendency of younger siblings on day 10 to have higher baseline corticosterone levels than older siblings (Fig. 6) can be explained by fat stores as the body condition of the youngest nestlings was particularly bad (Fig. 4). Hence, our prediction was confirmed that body energy stores predominantly determine both the adrenocortical response to a threat and baseline corticosterone levels in free-living birds with variable body conditions, rather than hatching order *per se*. Indeed, other studies of free-living nestlings also found no dependence of baseline and handling-induced corticosterone levels on hatching order (Sockman and Schwabl, 2001; Blas et al., 2005) and higher baseline corticosterone levels in junior collared dove *Streptopelia decaocto* nestlings with a much worse body condition than seniors (Eraud *et al.*, 2008).

In contrast to these studies on free-living birds, higher baseline and handling-induced corticosterone levels were found in older nestlings within the brood in captive, ad libitum fed canary *Serinus canaria* (Schwabl, 1999) and American kestrel nestlings (Love *et al.*, 2003b). Assuming that ad libitum fed nestlings all had a very good body condition, these studies may show an underlying pattern of decreasing corticosterone levels with hatching order. However, it seems that under natural conditions, body condition, which often varies systematically with hatching order, predominantly determines corticosterone levels and thus superimposes the underlying pattern shown in ad libitum fed birds. Because younger nestlings, when fed ad libitum, have lower corticosterone levels than their older siblings, but increase corticosterone levels when in bad body condition, there is often no or a negative relationship between corticosterone levels and hatching order in natural conditions. Hence the rank in corticosterone may be determined by the interaction between hatching order and body condition with body condition having a stronger effect. This is further supported by the significant interaction between hatching span and age difference to the oldest sibling in the analysis of total baseline corticosterone levels on day 21 (Table 2). Late-hatched nestlings in broods with a large hatching span had even higher corticosterone levels than late-hatched nestlings in broods with a small hatching span. A large hatching span presumably results in an increased competitive inferiority of last-hatched nestlings, a bad body condition and high total baseline corticosterone levels.

Love et al. (2003b) suggested that hierarchies in adrenocortical function among siblings may maintain developmental hierarchies. We argue that body condition is the stronger factor than hatching order. Although we found a significant correlation between the corticosterone rank on day 10 and 21 in siblings, only 26 – 34% kept their exact rank. Moreover, it was the order in corticosterone values imposed by body condition (younger siblings having higher corticosterone values) rather than the order of the supposed underlying pattern (younger siblings having lower corticosterone values) that was maintained over time in our study.

Conclusion

In this study we showed that the postnatal development of the adrenocortical response to stress depends on age and body condition, but not on brood hierarchy. We argue that this is an adaptive modulation of the adrenocortical stress response, because it can be related to potential costs (increased energy expenditure during stress only tolerable with sufficient energy stores) and potential benefits (defence ability). Therefore, the developmental hypothesis is a special case of the adaptive modulation of the adrenocortical response to stress. Adaptive modulations of the adrenocortical response to stress have been shown to occur as a response to many life cycle events and environmental conditions, e.g. harsh breeding conditions (Wingfield et al., 1995), the annual moult (Romero et al., 2005) and the value of the current brood (Lendvai and Chastel, 2008). The modulation of the adrenocortical response to stress during development is particularly important, because adverse effects of the stress response may shape the phenotype with respect to size, physiology and behaviour (Caldji et al., 2001; Kitaysky *et al.*, 2003; Müller at al., 2009b).

Another way of modulating the effects of an adrenocortical response to stress would be via a variation of CBG capacity, which would have the potential to buffer the released corticosterone and its deleterious effects in growing chicks, as found in an altricial passerine species (Wada *et al.*, 2007). However, in this study we found little evidence for a modulating role of CBG capacity during postnatal development. CBG capacity increased from day 10 to 21, but was unrelated to body condition, brood hierarchy or any other variable investigated, and thus had no significant effect on free corticosterone.

Acknowledgements

We warmly thank Ueli Schaffner, Renate Wullschleger, Martin Fischer, Martin Gassner, Linda Bischoff, Christophe Bonetti, Annette Sauter, Jodok Guntern, Matthias Ernst, Lena Senn, for their help in the field work and Ester Pellegrini and Moni Pfunder for the sex determination. Bettina Almasi and Martin Grüebler gave valuable comments on an earlier version of the manuscript. Financial support for this study was provided by the Swiss National Science Foundation Grant 3100A0-104134 to L.J and S.J-E.

References

Barsano, C.P., Baumann, G., 1989. Simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria; or how to calculate bound and free hormone? Endocrinology 124, 1101-1106.

Blas, J., Baos, R., Bortolotti, G.R., Marchant, T., Hiraldo, F., 2005. A multi-tier approach to identifying environmental stress in altricial nestling birds. Functional Ecology 19, 315-322.

Blas, J., Baos, R., Bortolotti, G.R., Marchant, T.A., Hiraldo, F., 2006. Age-related variation in the adrenocortical response to stress in nestling white storks (*Ciconia ciconia*) supports the developmental hypothesis. General and Comparative Endocrinology 148, 172-180.

Breuner, C.W., Orchinik, M., 2002. Plasma binding proteins as mediators of corticosteroid action in vertebrates. Journal of Endocrinology 175, 99-112.

Breuner, C.W., Orchinik, M., Hahn, T.P., Meddle, S.L., Moore, I.T., Owen-Ashley, N.T., Sperry, T.S., Wingfield, J.C., 2003. Differential mechanisms for regulation of the stress response across latitudinal gradients. American Journal of Physiology 285, R594-R600.

Caldji, C., Liu, D., Sharma, S., Diorio, J., Francis, D., Meaney, M.J., Plotsky, P.M., 2001. Development of individual differences in behavioral and endocrine responses to stress: role of the postnatal environment. In: McEwen, B.S., Goodman, H.M. (Eds.), Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms Oxford University Press, New York, pp. 271-292.

Carsia, R.V., Harvey, S., 2000. Adrenals. In: Whittow, G.C. (Ed.), Sturkie's Avian Physiology. Academic Press, San Diego, pp. 489-537.

DuRant, S.E., Romero, L.M., Talent, L.G., Hopkins, W.A., 2008. Effect of exogenous corticosterone on respiration in a reptile. General and Comparative Endocrinology 156, 126-133.

Eraud, C., Trouve, C., Dano, S., Chastel, O., Faivre, B., 2008. Competition for resources modulates cell-mediated immunity and stress hormone level in nestling collared doves (*Streptopelia decaocto*). General and Comparative Endocrinology 155, 542-551.

Fridinger, R.W., O'Reilly, K.M., an Kildaw, S., Loren Buck, C., 2007. Removal of a nest-mate elicits an age-dependent increase in plasma corticosterone of nestling Black-legged Kittiwakes. Journal of Field Ornithology 78, 93-99.

Fridolfsson, A.K., Ellegren, H., 1999. A simple and universal method for molecular sexing of non-ratite birds. Journal of Avian Biology 30, 116-121.

Heath, J.A., Dufty, A.M., 1998. Body condition and the adrenal stress response in captive American kestrel juveniles. Physiological Zoology 71, 67-73.

Holmes, W.N., Redondo, J.L., Cronshaw, J., 1989. Changes in the adrenal steroidogenic responsiveness of the mallard duck (*Anas platyrhynchos*) during early post-natal development. Comparative Biochemistry and Physiology Part A: Physiology 92, 403-408.

Jenni, L., Jenni-Eiermann, S., Spina, F., Schwabl, H., 2000. Regulation of protein breakdown and adrenocortical response to stress in birds during migratory flight. American Journal of Physiology 278, R1182-R1189.

Jenni-Eiermann, S., Glaus, E., Gruebler, M., Schwabl, H., Jenni, L., 2008. Glucocorticoid response to food availability in breeding barn swallows (*Hirundo rustica*). General and Comparative Endocrinology 155, 558-565.

Kaiser, A., 1993. A new multi-category classification of subcutaneous fat deposits of songbirds. Journal of Field Ornithology 64, 246-255.

Kawasaki, E.S., 1990. Sample preparation from blood, cells, and other fluids. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols - A guide to methods and applications. Academic Press, New York, pp. 146-152.

Kitaysky, A.S., Kitaiskaia, E.V., Piatt, J.F., Wingfield, J.C., 2003. Benefits and costs of increased levels of corticosterone in seabird chicks. Hormones and Behavior 43, 140-149.

Kitaysky, A.S., Kitaiskaia, E.V., Wingfield, J.C., Piatt, J.F., 2001. Dietary restriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. Journal of Comparative Physiology - B, Biochemical, Systemic, and Environmental Physiology 171, 701-709.

Kitaysky, A.S., Piatt, J.F., Wingfield, J.C., Romano, M., 1999. The adrenocortical stress-response of Black-legged Kittiwake chicks in relation to dietary restrictions. Journal of Comparative Physiology - B, Biochemical, Systemic, and Environmental Physiology 169, 303-310.

Lendvai, A.Z., Chastel, O., 2008. Experimental mate-removal increases the stress response of female house sparrows: The effects of offspring value. Hormones and Behavior 53, 395-401.

Love, O.P., Bird, D.M., Shutt, L.J., 2003a. Corticosterone levels during post-natal development in captive American kestrels (*Falco sparverius*). General and Comparative Endocrinology 130, 135-141.

Love, O.P., Bird, D.M., Shutt, L.J., 2003b. Plasma corticosterone in American kestrel siblings: Effects of age, hatching order, and hatching asynchrony. Hormones and Behavior 43, 480-488.

Love, O.P., Williams, T.D., 2008. Plasticity in the adrenocortical response of a free-living vertebrate: The role of pre- and post-natal developmental stress. Hormones and Behavior 54, 496-505.

Müller, C., Almasi, B., Roulin, A., Breuner, C.W., Jenni-Eiermann, S., Jenni, L., 2009a. Effects of corticosterone pellets on baseline and stress-induced corticosterone and corticosteroid-binding-globulin. General and Comparative Endocrinology 160, 59-66.

Müller, C., Jenni-Eiermann, S., Blondel, J., Perret, P., Caro, S.P., Lambrechts, M., Jenni, L., 2006. Effect of human presence and handling on circulating corticosterone levels in breeding blue tits (*Parus caeruleus*). General and Comparative Endocrinology 148, 163-171.

Müller, C., Jenni-Eiermann, S., Jenni, L., 2009b. Effects of a short period of elevated circulating corticosterone on postnatal growth in free-living Eurasian kestrels Falco tinnunculus. Journal of Experimental Biology 212, 1405-1412.

Munro, C., Stabenfeldt, G., 1984. Development of a microtitre plate enzyme immunoassay for the determination of progesterone. Journal of Endocrinology 101, 41-49.

Munro, C.J., Lasley, B.L., 1988. Non-radiometric methods for immunoassay of steroid hormones. In: Albertson, B.D., Haseltine, F.P. (Eds.), Non-radiometric assays : technology and application in polypeptide and steroid hormone detection. Alan R. Liss, Inc, cop., New York, pp. 289-329.

Payne, R.W., 2003. The Guide to GenStat Release 7.1., Part 2: Statistics. VSN International, Oxford.

Ramos-Fernandez, G., Nunez-de la Mora, A., Wingfield, J.C., Drummond, H., 2000. Endocrine correlates of dominance in chicks of the blue-footed booby (*Sula nebouxii*): Testing the Challenge Hypothesis. Ethology, Ecology and Evolution 12, 27-34.

Romero, L.M., Strochlic, D., Wingfield, J.C., 2005. Corticosterone inhibits feather growth: Potential mechanism explaining seasonal down regulation of corticosterone during molt. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 142, 65-73.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21, 55-89.

Schwabl, H., 1999. Developmental changes and among-sibling variation of corticosterone levels in an altricial avian species. General and Comparative Endocrinology 116, 403-408.

Sims, C.G., Holberton, R.L., 2000. Development of the corticosterone stress response in young Northern Mockingbirds (*Mimus polyglottos*). General and Comparative Endocrinology 119, 193-201.

Sockman, K.W., Schwabl, H., 2001. Plasma corticosterone in nestling American kestrels: Effects of age, handling stress, yolk androgens, and body condition. General and Comparative Endocrinology 122, 205-212.

Thompson, R., Welham, S.J., 2003. REML analysis of mixed models. In: Payne, R.W. (Ed.), The Guide to GenStat Release 7.1., Part 2: Statistics VSN International, Oxford, pp. 513-607.

Wada, H., Hahn, T.P., Breuner, C.W., 2007. Development of stress reactivity in white-crowned sparrow nestlings: Total corticosterone response increases with age, while free corticosterone response remains low. General and Comparative Endocrinology 150, 405-413.

Walker, B.G., Wingfield, J.C., Boersma, P.D., 2005. Age and Food Deprivation Affects Expression of the Glucocorticosteroid Stress Response in Magellanic Penguin (*Spheniscus magellanicus*) Chicks . Physiological and Biochemical Zoology 78, 78-89.

Wiehn, J., Ilmonen, P., Korpimäki, E., Pahkala, M., Wiebe, K.L., 2000. Hatching asynchrony in the Eurasian kestrel *Falco tinnunculus*: An experimental test of the brood reduction hypothesis. Journal of Animal Ecology 69, 85-95.

Wingfield, J.C., O'Reilly, K.M., Astheimer, L.B., 1995. Modulation of the adrenocortical responses to acute stress in Arctic birds: A possible ecological basis. American Zoologist 35, 285-294.

Chapter 4

Strategies of the last-hatched nestling under varying food availability in asynchronous broods of the Eurasian kestrel: corticosterone, condition and growth

Claudia Müller, Susanne Jenni-Eiermann and Lukas Jenni

Abstract

Hatching asynchrony is a widespread phenomenon in altricial birds and is often explained with the brood reduction hypothesis, which postulates that brood size is adaptively reduced under conditions of food scarcity through starvation death of the last-hatched, least competitive nestling for the benefit of a higher fledging success of its older siblings. In this study we investigate the strategies adopted by the last-hatched nestlings, rather than the improvement of fitness of the adults, as in other studies. We postulate that last-hatched nestlings should try to catch-up in growth with older siblings under good conditions, but would be the first to have to switch to a survival mode under bad conditions. Glucocorticoids are candidate hormones to mediate such strategies. In free-living Eurasian kestrel *Falco tinnunculus* nestlings we measured growth, baseline total and free corticosterone levels, the adrenocortical response to handling and body energy stores under varying food conditions (yearly fluctuations in prey abundance and short-term impairment of hunting success of adults by rain). We showed that last-hatched nestlings under good conditions (no rain, large furcular fat stores) had lower baseline corticosterone levels and an higher wing feather growth rate than older siblings, indicating catch-up growth, enabled by very low corticosterone levels. Short-term food restriction (rain) resulted in higher baseline corticosterone levels in last-hatched nestlings than in older siblings. Furcular fat stores were lower in last-hatched nestlings compared with older siblings. Growth of wing feathers and body mass was reduced and mortality increased in last-hatched nestlings with high corticosterone levels. We conclude that last-hatched nestlings modulate baseline and handling-induced corticosterone levels and growth allocation compared with older siblings. This modulation is condition-dependent and varied with food conditions. Last-hatched nestlings seem to follow an “all-or-nothing-strategy” by investing in structural growth and refraining from large body fat stores under good conditions to catch-up in growth. Under bad conditions, last-hatched nestlings have to switch more rapidly to a survival mode because of their small body energy stores.

1. Introduction

Hatching asynchrony, an extended hatching period, is a widespread phenomenon in altricial birds. Lack (1954) explained hatching asynchrony with the brood reduction hypothesis, i.e. the adaptive reduction of brood size under conditions of food scarcity. Clutch size can be adapted to the prevailing conditions, but especially in large species with long incubation and nestling periods, this decision has to been made long before maximal food demand of nestlings is reached. Food availability during the nestling phase often is not predictable at the time of laying. Food availability can vary because of short-term perturbations (e.g. bad weather) or on a longer-term scale such as between years (e.g. vole cycles). Good conditions allow a maximum of fledglings to be raised, but under bad conditions brood size is efficiently reduced through starvation of the last-hatched and least-competitive, which improves the fledging success of the older siblings. Many additional hypotheses explaining hatching asynchrony have been proposed (reviewed e.g. in Magrath, 1990; Stenning, 1996), demonstrating that other selective pressures (varying between and within species) also can result in hatching asynchrony. However, the brood reduction hypothesis is still the main explanation for hatching asynchrony and has been extended by theoreticians (e.g. Pijanowski, 1992). Hatching asynchrony has been shown to be adaptive in different species (e.g. Ricklefs, 1965; Husby, 1986; Valkama et al., 2002; but not in Hillstrom and Olsson, 1994; Wiehn et al., 2000).

Under the brood reduction hypothesis, hatching asynchrony entails an aggravated parent-offspring conflict (Forbes, 1993) with the last-hatched nestling which is most prone to be sacrificed under suboptimal environmental conditions for the benefit of its siblings and an overall higher fitness of the parents. While most studies focused on the fitness of the parents, the perspective of the last-hatched nestling received less attention. Under bad environmental conditions, a reduced competitive ability of the last-hatched nestling exposes it to be the first to die from starvation. However, if conditions allow the last-hatched to survive, strategies are likely which ameliorate its situation and enable it to, at best, catch up with its older siblings. To catch up with older siblings is important in order to be competitive in the nest and fledge at about the same time as the older siblings. One possibility of the last-hatched to catch up with siblings is an adaptation of the allocation of energy and nutrients, i.e. to favour structural and feather growth at the expense of building up body energy stores for periods of reduced food availability (e.g. bad weather periods). Last-hatched nestlings could follow an “all-or-nothing” strategy. If food is plentiful, last-hatched nestlings would refrain from building up large body energy stores, but would invest in structural growth, particularly growth of wing feathers. If food availability is limited, last-hatched nestlings would suffer first and, because of little body energy stores, would soon have to physiologically switch into a survival mode and restrict growth severely. Presumably, they would be the first to starve.

Glucocorticoids, corticosterone in birds, play an important role in the trade-off between immediate survival and growth. Elevated corticosterone levels mobilize stored energy by proteolysis and increasing gluconeogenesis and suppress anabolic processes like growth (e.g. reviewed in

Wingfield et al., 1998; Carsia and Harvey, 2000). Thus, circulating corticosterone may differentially regulate energy allocation in nestlings depending on their rank in brood hierarchy. Indeed, baseline levels of circulating corticosterone have been shown to vary with the rank under *ad libitum* food conditions, being lowest in the last-hatched nestling (Schwabl, 1999; Love et al., 2003). However, in free-living nestlings exposed to variable food intake, higher corticosterone levels in last-hatched nestlings being in a worse condition were found (Eraud et al., 2008) or no dependence of corticosterone on brood hierarchy (Sockman and Schwabl, 2001; Blas et al., 2005). A strategy of the last-hatched nestling under good conditions could therefore be to have lower baseline corticosterone levels than its older siblings, which would enable to grow faster and to catch up with siblings. Under bad conditions corticosterone would increase faster in last-hatched nestlings because of their smaller body energy stores and growth would be restricted or stopped rapidly.

The amount of body energy stores may in turn modulate the adrenocortical response to an acute stressor which is an energetically demanding process (DuRant et al., 2008). The adrenocortical response to an acute stressor may be dampened under low food availability to avoid the energetic costs (Müller et al., submitted). Thus, we expect last-hatched nestlings to show a dampened adrenocortical response to an acute stressor, because they normally have less body energy stores than older siblings.

The aim of this paper was to investigate whether the strategy of growth and glucocorticoid response to an acute and chronic stressor in last-hatched nestlings differs from that of older siblings under natural conditions of varying food availability. We chose a nest box population of a small, semi-altricial raptor species, the Eurasian kestrel *Falco tinnunculus*, whose nestlings hatch asynchronously and mortality occurs during the middle of the nestling period when food is scarce (Wiehn et al., 2000). Its main prey, the common vole *Microtus arvalis*, shows population cycles, resulting in food availability varying greatly between years. At a higher temporal resolution, rain negatively affects hunting performance of adults (Cavé, 1968; personal obs.) and provokes short-term reductions in food availability. We first examined whether year and rain as proxys for varying food availability at the scale of the year and the scale of days, respectively, affect growth rates of nestlings. By inspecting the amount of subcutaneous fat stores of nestlings, we could assess the amount of body energy stores.

We made the following predictions, based on the arguments above, about baseline corticosterone levels, growth rate and the adrenocortical response to an acute stressor (handling by the experimenter) of last-hatched nestlings compared with older siblings. (1) Under good environmental conditions (good year, no rain) we predicted lower baseline corticosterone levels, a similarly high or higher growth rate (predominantly in wing feathers) and a similar adrenocortical response to an acute stressor in last-hatched nestlings compared with their older siblings. (2) In a year with low food availability (long-term food-shortage), we expect elevated baseline corticosterone levels, a reduced growth rate and a reduced adrenocortical response to handling in all nestlings of a brood compared with a year with higher food availability. It is to be expected that the limited food

availability causes the death of the last-hatched and a chronic food-stress in the remaining nestlings. (3) During an episodic food shortage by rain, we expect higher corticosterone levels (emergency life history stage through energy shortage), a slowed-down growth and a reduced adrenocortical response to handling in the last-hatched nestlings compared with the older siblings. We expect this, because last-hatched nestlings probably have smaller energy stores than older siblings. Taking all situations together, we expect body energy stores to decrease with hatching order, baseline corticosterone levels to be negatively related to body energy stores, and the adrenocortical response to handling to be positively related to body energy stores. In last-hatched nestlings with large body energy stores, we predict baseline corticosterone levels to be lower than in older siblings, similar as in lab studies (Schwabl, 1999; Love et al., 2003). Finally, high corticosterone levels should indicate oncoming mortality.

Because brood reduction normally occurs in the middle of the nestling period in this species (Wiehn et al., 2000), we expected larger within-brood differences concerning corticosterone and growth in the middle, rather than towards the end of the nestling period, when nestlings are near maximum body mass and later-hatched ones have compensated their backlog in body mass to a certain degree and are more competitive.

A further aspect of our study was that we not only measured total circulating corticosterone, but also the binding capacity of corticosterone binding globulins CBG, and thus were able to estimate free corticosterone levels, the fraction considered to be biologically active (Breuner and Orchinik, 2002). CBG has the potential to modulate circulating total corticosterone levels and has been shown to buffer total corticosterone levels during certain developmental stages (Wada et al., 2007).

2. Material and Methods

2.1. Study species and study sites

The Eurasian Kestrel *Falco tinnunculus* is a small raptor with reversed size-dimorphism. It breeds in open landscapes and hunts on small vertebrates (especially voles) and large insects. The female lays 3-7 eggs in two-day intervals and incubates them for 29 days, usually beginning after the third egg. This results in a partial hatching asynchrony. The three oldest nestlings within a brood have about the same age, while the others hatch at intervals of about 2 days. When food is scarce, the last-hatched chicks regularly die, usually in the first half of the nestling stage (own observation; Wiehn et al., 2000), when the food demand of the brood increases. When food is scarce, access of the last-hatched nestlings to the feeding female is blocked by the older siblings and they starve, but later-hatched nestlings are not attacked by older siblings and no siblicide occurs as in other species (e.g. Newton, 1979; Tarlow et al., 2001; Legge, 2002). The nestlings stay in the nest for 32 to 39 days.

The field work was performed in the Jura mountains and river valleys in North-western Switzerland (7°60'E / 47°30'N), where Eurasian kestrels breed in nest boxes mounted on agricultural buildings in open rural landscapes. In 2004 and 2005, 294 nestlings of 61 broods within an area covering approximately 600 km^2 were investigated.

2.2. Nest box controls and age determination

From April onwards, nest boxes were controlled biweekly for clutches and every second day before hatching to determine the hatching date of the oldest nestlings. Later-hatched siblings were aged by a combination of these regular controls and the wing length at day 10. From the individual nestling age (in days) of each nestling, we calculated the age difference to the oldest siblings (in days, 0d: 118 nestlings, -1 day: 98 nestlings; -2 days: 43 nestlings, -3 days: 13 nestlings, -4 days: 11 nestlings, -5 days: 2 nestlings) and determined the last-hatched nestling of the brood. Brood size ranged from 1 to 7 nestlings.

2.3. Blood sampling

Blood samples were taken when the oldest nestlings were about 10 and 21 days old. For logistical reasons, not all broods could be sampled on the intended age and the age of the oldest nestlings ranged from 9 to 11 (54 of 66 broods sampled on day 10) and from 20 to 23 days (51 of 65 broods sampled on day 21), respectively. We checked for an effect of taking the samples earlier or later than intended on all analyses, but because there was no significant effect, we excluded this parameter.

For blood sampling, 1 - 3 nestlings were removed from the nest box and blood was taken if possible within 3 minutes (mean $\pm$ SD: 2.37 $\pm$ 1.06 min, n = 524) by puncturing the alar vein. When brood size was large and nestlings defended themselves (especially on day 21), blood sampling was completed only after up to 6 min after capture. Corticosterone levels increased significantly with time after capture. This increase was small in 10 days old nestlings (slope 1.2 ng/ml corticosterone / min, R^2 = 0.02, n = 280) and more pronounced in 21 days old nestlings (slope 1.97 ng/ml corticosterone / min, R^2 = 0.14, n = 244). Because most of the samples were taken within 3 min (84% of the samples on day 10, 67% on day 21) and the increase with time after capture was not strong, we included all samples in the analysis and introduced time after capture as the first variable into the models to correct for this initial increase in corticosterone as response to capture. An analysis of samples taken within 3 min gave similar results.

After blood sampling, nestlings were measured and then held in a cloth bag. About 17 min after capture (17.06 $\pm$ 2.43 min, 14 – 21 min, n = 487), a second, handling-induced blood sample was taken. After returning this first group of nestlings to the nest box, the second batch of the brood was blood sampled and measured, and in large broods a third batch. On day 21, but not on

day 10, batches 2 and 3 showed higher corticosterone levels than the first batch, probably due to our presence at the nest (Müller et al., submitted). We therefore introduced batch number as a factor into the models dealing with data from day 21 to correct for this disturbance effect.

Blood was collected with heparinized capillary tubes (80 ul blood per sample), immediately transferred to Eppendorf tubes and centrifuged within 1 h. Plasma and blood cells were separated and stored in liquid nitrogen until transfer to the lab, where they were stored at –20°C until analysis. Plasma was used for corticosterone and CBG analysis, blood cells served for sex determination. Blood sampling was approved by the Cantonal committee for animal research (animal experiment permit n°274 from the Cantonal Veterinarian Office of Baselland).

2.4. Morphology, body condition and sex determination

At the age of 10, 13, 16, 21 and 25 days, we measured the length of primary 8 (second longest primary) to the nearest 0.5 mm, and body mass to the nearest g.

As in passerines (Kaiser, 1993), we assessed the visible subcutaneous fat stores at the furcula by assigning a fat score ranging from 0 to 4 (0: no visible fat; 1: 1 mm stripe of fat at the bottom of the furcular pit; 2: fat stripes 2-3 mm broad; 3: furcular pit nearly covered with fat (about 75%), 4: furcular pit completely filled with fat).

Nestlings were sexed with molecular methods by fragment analysis on CHD1W/CHD1Z (Fridolfsson and Ellegren, 1999) using blood cells of blood samples extracted with the QIAamp DNA extraction Kit (Qiagen) in 2004 and after Kawasaki (1990) in 2005. Samples of 2004 were analysed at the Swiss Federal Institute for Forest, Snow and Landscape Research in Birmensdorf, Switzerland and those of 2005 at the Agroscope Research Station ACW in Wädenswil, Switzerland.

2.5. Weather parameters

For each brood, we used the weather data of the nearest weather station (Basel, Rünenberg or Würenlingen, 1 – 20 km away) of the Federal Office of Meteorology and Climatology MeteoSwiss. For each brood the we checked, whether there was rainfall in the two days preceding nestling day 10 and 21 and during the four growth periods day 10 - 13, day 13 - 16, day 16 - 21 and day 21 - 25.

2.6. Corticosterone assay

Plasma corticosterone concentration was determined using an enzyme immuno assay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988). Corticosterone in 5 μl plasma and 195 μl water was extracted with 4 ml dichloromethane, re-dissolved in phosphate buffer and given in triplicates in the

enzyme immuno assay. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, Progesterone 0.004%, 18-OH-DOC 0.01%, Cortisol 0.12%, 18-OH-B 0.02% and Aldosterone 0.06%) was 1:8'000. HRP (1:400'000) linked to corticosterone served as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. Plasma pools from chickens with two different corticosterone concentrations were included as internal controls on each plate. If the concentration was below the detection threshold, the determination was repeated with 10 µl plasma. If the concentration was still below the detection threshold, the value of the lowest detectable concentration (1 ng ml^{-1}) was assigned (23 samples). Intra-assay variation ranged from 4.5 to 10.8 % and inter-assay variation from 9.6 to 17.6 %, depending on the concentration of the internal control and the year of determination.

2.7. Corticosteroid-binding-globulin

The affinity and capacity of corticosteroid-binding-globulin (CBG) was measured with a radioligand-binding assay with tritiated corticosterone following Breuner et al. (2003). For point sample analysis, 10-15 µl plasma were stripped of endogenous steroids with 2 parts of dextran-coated charcoal (0.1% dextran, 1% Norit A charcoal in 50 mM Tris) for 30 minutes at room temperature. Outside this stripping procedure, the plasma was maintained below 4°C. The final assay dilution of plasma samples was 1:99. The binding assay was carried out in 50 nM Tris buffer at 4°C and terminated after 2 h. 1 h before filtering, glass fiber filters (Whatman) were soaked in 25 nM Tris with 0.3% polyethylenimine. After filtration, filters were rapidly rinsed with 3 rinses of 3 ml ice-cold 25 nM Tris. Point sample analysis was performed with individual plasma samples, for the saturation analysis pooled samples were run. For the saturation analyses, 0.25-12 nM [^{3}H]Corticosterone were incubated with pooled plasma with and without 1µM unlabeled corticosterone. 20 nM [^{3}H]corticosterone was employed to estimate CBG capacity in individual birds. Affinity estimates obtained from equilibrium saturation analysis (Kd = 4.59, see Müller et al., 2009a) indicated that this ligand concentration occupies ~80% of total binding sites. For analysis, the samples were corrected to 100 % capacity within each assay. The intra-assay variation was 7.1 %, the inter-assay variation 7.7 %.

The equation of Barsano and Baumann (1989) was used to estimate free corticosterone titers from total corticosterone concentrations and CBG binding parameters

$$H_{free} = 0.5 \times \left[H_{total} - B_{max} - 1/K_a\right] \pm \sqrt{(B_{max} - H_{total} + 1/K_a)^2 + 4(H_{total}/K_a)}$$

where H_{free} is free Hormone, H_{total} is total Hormone, B_{max} is total binding capacity of CBG, and K_a = 1/dissociation constant (K_d) (all values in nM).

2.8. Statistical analysis

We performed mixed model analyses in Genstat 9 (Thompson and Welham, 2003; Payne, 2003) and t-Tests in SPSS 12. Brood was introduced as random factor in all mixed models to correct for non-independence of siblings. Baseline total and free corticosterone and CBG capacity were square-root-transformed to obtain normally distributed residuals.

Brood parameters (clutch size, number of nestlings, number of fledglings, fledging success) in the study years 2004 and 2005 were compared with t-Tests. Nestling growth parameters were assessed for year differences in different mixed models with primary length, body mass and fat score on day 10 and 21 as dependent variables and year as fixed factor. Effects of rain on primary and body mass growth rates were evaluated in mixed models with rain (rain or no rain during the respective growth period) as fixed factor.

Dependence of baseline total and free corticosterone levels, handling-induced total corticosterone levels and CBG capacity on the fixed factors year, age difference to the oldest sibling within the brood, being the last-hatched of the brood, sex and rain were assessed in different mixed models. As discussed above, we introduced time after capture and batch number (day 21) as the first parameters into the mixed models. Mean baseline corticosterone levels in figures and in text are given corrected for a mean time after capture of 2 min, according to the slope of the regression between baseline corticosterone levels and time after capture.

Dependence of furcular fat score from the age difference to the oldest sibling and being the last-hatched of the brood was assessed in a mixed model on day 10 and 21.

The dependence of primary growth rate and body mass increase on individual nestling age, baseline total corticosterone level (square-root transformed and corrected for time after capture), and being the last-hatched nestling of the brood and their interaction were tested in different mixed models .

The relationship between survival and baseline corticosterone (square-root transformed and corrected for time after capture) was investigated in generalized linear mixed models with a binomial distribution, with corticosterone, being the last-hatched and their interaction as fixed effects.

3. Results

3.1. Effects of year and rain on growth and survival

As a prerequisite for further analyses, we first examined whether conditions for nestlings differed between the two study years and varied with rain, in order to ascertain that year and rain can be used as proxys for different growth conditions.

Breeding density was higher in 2005 (72 broods in our nest boxes) than in 2004 (57 broods). There was no significant difference in clutch size, number of nestlings, number of fledglings and fledging success between years (Table 1). However, in 2005 nestlings grew faster than in 2004. On day 10 and 21, nestlings had significantly longer primaries, a larger body mass and more furcular fat stores in 2005 than in 2004, with the exception of body mass on day 21, which did not differ between 2004 and 2005 (Table 1). Mortality of nestlings between day 10 and 25 was higher in 2004 (21 of 142 or 14.8% of nestlings died) than in 2005 (14 of 144 or 9.7% died), but this difference was not significant (χ^2 = 1.71, d.f. = 1, p= 0.191).

Table 1. Key parameters of broods and nestlings in 2004 and 2005. Brood parameters were compared between years with a t-test and nestling parameters in a mixed model (with brood identity as random factor).

	2004	2005	Test-statistic	Prob.
Brood parameters	n = 57	n = 72		
Clutch size	4.96 ± 0.13	4.79 ± 0.12	1.008	0.316
Number of nestlings	4.39 ± 0.23	4.04 ± 0.19	1.157	0.249
Number of fledglings	3.49 ± 0.24	3.42 ± 0.21	0.232	0.817
Fledglings / nestlings	0.79 ± 0.03	0.84 ±0.03	-1.24	0.219
Nestling parameters	n = 142	n = 152		
Primary length on day 10	7.20 ± 0.61	11.97 ± 0.50	13.71	<0.001
Primary length on day 21	70.2 ± 1.1	80.2 ± 0.9	20.67	<0.001
Body mass on day 10	103.7 ± 2.5	122.0 ± 2.1	12.89	<0.001
Body mass on day 21	204.4 ± 2.3	211.9 ± 2.1	2.36	0.124
Fat score on day 10	1.00 ± 0.06	1.22 ± 0.04	5.47	0.019
Fat score on day 21	2.35 ± 0.07	2.81 ± 0.05	11.44	<0.001

Primary growth rate from day 13 to 16 was reduced if there was rain during this period (Wald = 5.42, d.f. = 1, p = 0.020). Otherwise, we did not find significant negative impacts of rain on growth rates.

3.2. Corticosterone levels in last-hatched nestlings depending on environmental conditions

3.2.1. Effect of year

We tested whether year and rain (as proxys of variation in environmental conditions) affected baseline corticosterone levels (total and free) as well as handling-induced corticosterone levels on day 10 (Table 2) and on day 21 (Table 3) and whether these effects differed between last-hatched and older nestlings. We corrected for time after capture and included brood size and age difference

Table 2. Dependence of baseline total and free corticosterone, CBG-capacity and handling-induced (17 min after capture) corticosterone in kestrel nestlings on various parameters related to the brood and the nestling, year and rain on day 8-9 (fixed factors in a mixed model with brood identity as random factor) for day 10 of age. Significant effects are highlighted in bold.

Nestling day 10		Baseline						Handling-induced	
		Total cort (n=279)		Free cort (n=249)		CBG cap. (n=249)		Total cort (n=245)	
Explanatory variable	d.f.	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p
Time after capture [min]	1	11.08	<0.001	4.74	**0.029**	0.41	0.521	0.06	0.806
Year	1	0.24	0.622	0.21	0.648	8.83	**0.003**	5.38	**0.020**
Brood size	1	0.50	0.481	0.13	0.721	5.82	**0.016**	0.81	0.368
Age difference to the oldest sibling [d]	1	1.87	0.171	3.06	0.080	0.83	0.362	0.00	0.975
Last-hatched nestling of the brood	1	2.13	0.144	1.59	0.207	0.42	0.515	0.61	0.435
Sex	1	0.09	0.758	0.08	0.781	0.00	0.966	1.25	0.263
Rain day 8-9 [yes/no]	1	5.02	**0.025**	2.49	0.114	0.51	0.474	5.52	**0.019**
Age difference x Year	1	1.72	0.190	0.88	0.348	0.28	0.596	0.05	0.820
Last-hatched nestling x Year	1	1.58	0.209	2.33	0.127	1.54	0.215	4.77	**0.029**
Age difference x Rain day 8-9	1	1.66	0.197	5.03	**0.025**	0.79	0.375	7.78	**0.005**
Last-hatched nestling x Rain day 8-9	1	4.22	**0.040**	5.90	**0.015**	0.09	0.762	1.78	0.182
Age difference x Sex	1	1.06	0.304	2.46	0.117	0.59	0.441	0.30	0.584
Last-hatched nestling x Sex	1	0.19	0.662	0.02	0.879	0.08	0.778	1.36	0.243

Table 3. Dependence of baseline total and free corticosterone, CBG-capacity and handling-induced (17 min after capture) corticosterone in kestrel nestlings on various parameters related to the brood and the nestling, year and rain on day 19-20 (fixed factors in a mixed model with brood identity as random factor) for day 21 of age. Significant effects are highlighted in bold.

Nestling day 21		Baseline						Handling-induced	
		Total cort (n=245)		Free cort (n=234)		CBG cap. (n=234)		Total cort (n=243)	
Explanatory variable	d.f.	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p
Time after capture [min]	1	66.83	<0.001	20.04	<0.001	0.45	0.503	0.35	0.557
Batch number	2	23.92	<0.001	8.17	0.017	2.82	0.245	-	-
Year	1	7.33	0.007	0.74	0.388	6.92	0.009	9.45	0.002
Brood size	1	3.13	0.077	0.78	0.379	0.41	0.523	0.97	0.325
Age difference to the oldest sibling [d]	1	0.20	0.654	0.02	0.882	0.04	0.843	0.06	0.812
Last-hatched nestling of the brood	1	0.42	0.516	0.61	0.435	0.16	0.692	0.19	0.661
Sex	1	1.76	0.185	1.47	0.226	0.07	0.798	3.16	0.075
Rain day 19-20 [yes/no]	1	0.03	0.872	0.22	0.636	0.20	0.658	0.00	0.966
Age difference x Year	1	0.01	0.938	0.12	0.727	0.01	0.935	5.41	0.020
Last-hatched nestling x Year	1	1.01	0.314	0.82	0.364	0.00	0.946	0.24	0.627
Age difference x Rain day 19-20	1	0.03	0.854	0.50	0.481	0.51	0.475	1.98	0.159
Last-hatched nestling x Rain day 19-20	1	0.65	0.422	0.31	0.580	0.28	0.596	0.52	0.469
Age difference x Sex	1	0.52	0.469	0.34	0.563	2.13	0.144	3.02	0.082
Youngest of the brood x Sex	1	0.15	0.698	0.42	0.518	0.07	0.789	0.28	0.594

to the oldest sibling into the model in order to differentiate whether effects were due to being younger or being the last-hatched in the nest.

Baseline total corticosterone on day 21, but not on day 10, was higher in 2005 than in 2004 (Table 3; mean ± SE: 6.05 ± 0.39 ng/ml, n = 122, in 2004 and 8.49 ± 0.55 ng/ml, n = 133, in 2005; means corrected for a time after capture of 2 min). On day 10, but not on day 21, baseline total corticosterone was higher in the last-hatched nestling than in older siblings (Table 2; 9.47± 1.57 ng/ml, n = 59, in last-hatched nestlings versus 6.38 ± 0.36 ng/ml, n = 221, in older siblings). Baseline total or free corticosterone did not vary with the within-brood age.

CBG capacity was higher in 2005 than in 2004 on day 10 and 21, but there was no effect of within-brood age and last-hatched nestling (Table 2 and 3, day 10: 2004: 55.8 ± 2.5 nmol, n = 113; 2005: 68.7 ± 3.1 nmol, n = 136; day 21: 2004: 70.8 ± 3.0 nmol, n = 109; 2005: 83.7 ± 3.6 nmol, n = 136).

Handling-induced total corticosterone was higher in 2005 than in 2004 on both day 10 and day 21, (Table 2 and 3, day 10: 2004: 18.30 ±0.79 ng/ml, n = 108; 2005: 21.81 ± 0.83 ng/ml, n = 137; day 21: 2004: 21.62 ± 0.84 ng/ml, n = 111; 2005: 27.02 ± 1.05 ng/ml, n = 130). On day 10, the last-hatched nestling, independent of its within-brood age (Age difference to the oldest sibling and

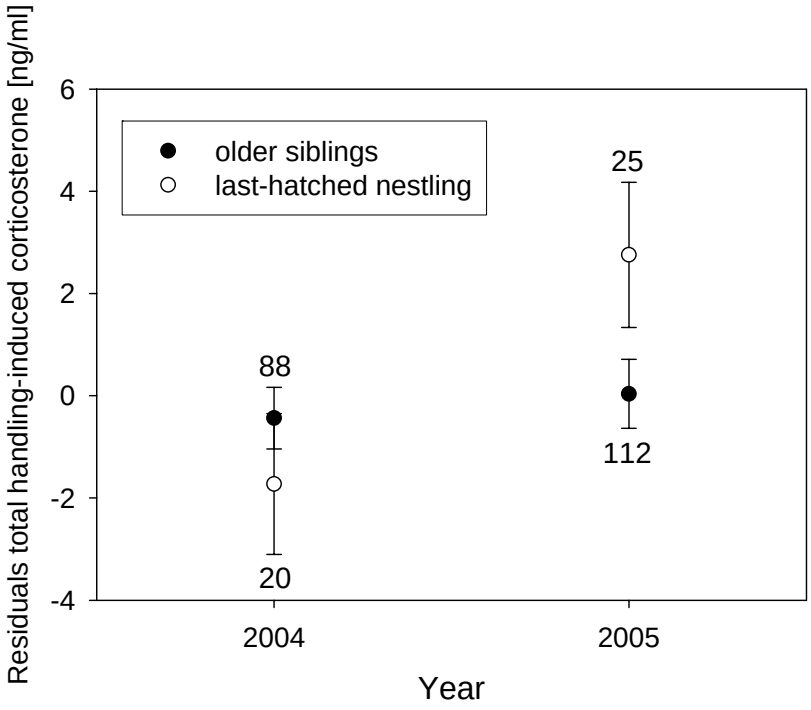

Fig. 1. Mean total handling-induced corticosterone levels (± SE) on day 10 for the two study years and for last-hatched nestlings and older siblings. Total handling-induced corticosterone levels were measured 17 min after capture and are given as residuals from a mixed model analysis with the variables Time after capture, Brood size, Age difference to the oldest sibling, Sex, Rain day 8-9 and interactions (corresponding to Table 1 but without the Parameters Year and Being the last-hatched nestling). Numbers near error bars indicate sample sizes.

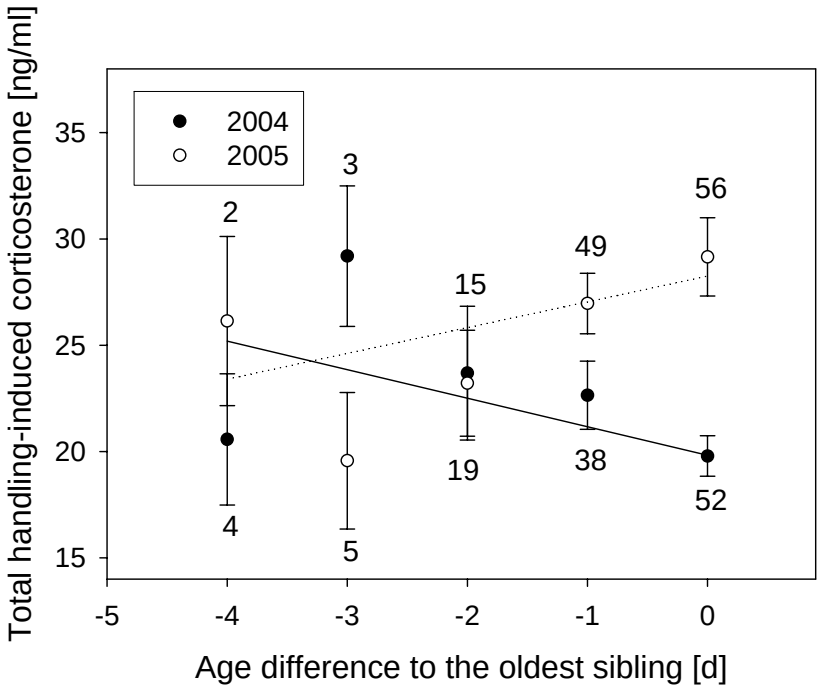

Fig. 2. Mean total handling-induced corticosterone levels (± SE) on day 21 plotted against the age difference to the oldest sibling for the two study years. Lines represent the estimated corticosterone levels from the mixed model in Table 3. Numbers near error bars indicate sample sizes.

its interaction with Year not significant), showed lower handling-induced corticosterone levels in 2004 and higher handling-induced corticosterone levels in 2005 compared with older siblings (interaction Last-hatched nestling x Year significant, Table 2, Fig. 1). On day 21, handling-induced corticosterone in older nestlings within the brood was higher in 2005 than in 2004 (interaction Age difference to the oldest sibling x Year significant, Table 3, Fig. 2).

3.2.2. Effect of rain

If it rained during the two preceding days, baseline total corticosterone levels on day 10 were significantly higher than without rain (Table 2, without rain: 5.53 ± 0.46 ng/ml, n = 108; with rain: 7.64 ± 0.57 ng/ml, n = 171). Additionally, with rain baseline free corticosterone levels on day 10 were higher in younger nestlings than in the older siblings (Age difference to the oldest sibling x Rain day 8-9 significant in free corticosterone). When it rained, both total and free corticosterone levels were particularly high in the last-hatched nestling within a brood (Last-hatched nestling x Rain day 8-9 significant, Table 2, Fig. 3, free corticosterone of last-hatched versus older nestlings $t = -2.56$, d.f. = 145, $p = 0.012$). Without rain during the two preceding days, baseline free corticosterone was significantly lower in last-hatched nestlings compared with older siblings ($t = 2.14$, d.f. = 99.2, $p = 0.035$), while this difference was not significant in baseline total corticosterone ($t = 1.01$, d.f. = 41.415, $p = 0.319$).

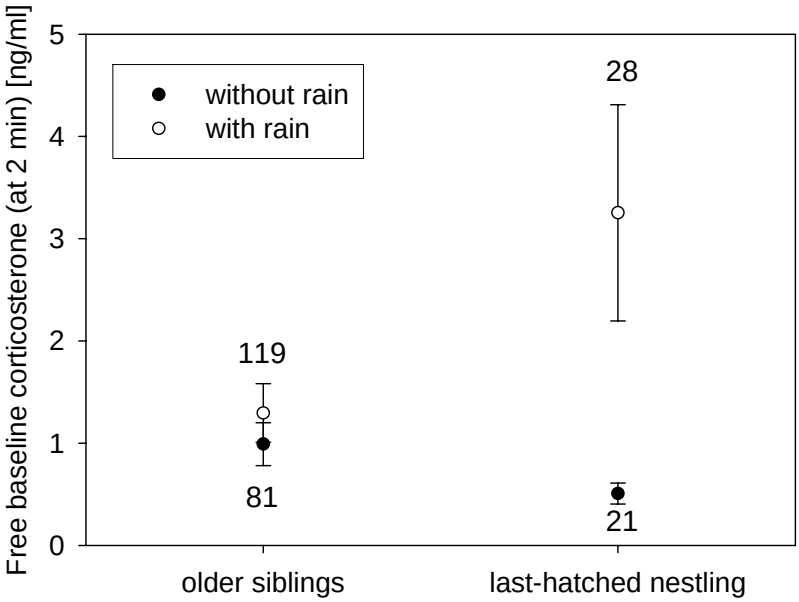

Fig. 3. Mean free baseline corticosterone levels (± SE) on day 10 of last-hatched nestlings and their older siblings with and without rain during the two preceding days. Free baseline corticosterone levels have been corrected for a time after capture of 2 min (the mean time after capture) according to the slope from the model given in Table 2. Numbers near error bars indicate sample sizes.Handling-induced corticosterone levels on day 10 were higher if it rained during the two preceding days (Table 2, without rain: 18.13 ± 0.90 ng/ml, n = 96; with rain: 21.63 ± 0.76, n = 149), particularly in younger nestlings (interaction Age difference to the oldest sibling x rain significant, Table 2).

3.3. Corticosterone levels in last-hatched nestlings depending on body fat stores

Conditions during growth translate into the amount of body energy stores (e.g. fat stores) of nestlings. Therefore, we also examined whether corticosterone levels were correlated with body fat stores and whether this relationship differed between last-hatched and older nestlings.

On day 10, furcular fat score was related to the within-brood age difference with later-hatched nestlings having smaller fat stores than older siblings (Wald = 73.02, d.f. = 1, $p < 0.001$). After correcting for this within-brood age effect the youngest nestling of the brood had disproportionally smaller fat stores (Wald = 5.94, d.f. = 1, $p = 0.015$, Fig. 4). On day 21, furcular fat score was independent of the within-brood age difference (Wald =2.20, d.f. = 1, $p = 0.138$) and of being the last-hatched of the brood (Wald = 0.01, d.f. = 1, $p = 0.929$).

On day 10, baseline total and free corticosterone levels were negatively correlated with furcular fat score, while the increase in corticosterone to handling was positively correlated with furcular fat score (Table 4). For baseline total corticosterone levels (Fig. 5), the relationship with fat score was significantly more pronounced in the last-hatched nestlings than in the older siblings (interaction Furcular fat score x Youngest of the brood significant), independently of the within-brood age difference (interaction Furcular fat score x Age difference not significant). The few last-

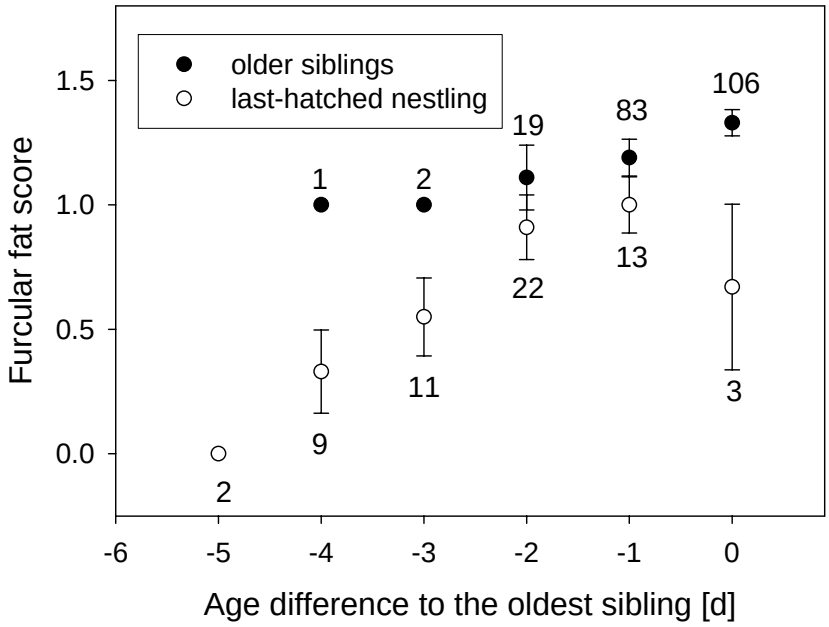

Fig. 4. Mean furcular fat score (± SE) on day 10 plotted against the age difference to the oldest sibling and the nestling status within the brood (last-hatched versus older siblings). Numbers near error bars indicate sample sizes. The three last-hatched nestlings with age difference 0 were from small broods, whose last-hatched nestlings hatched on the same day as their oldest siblings.

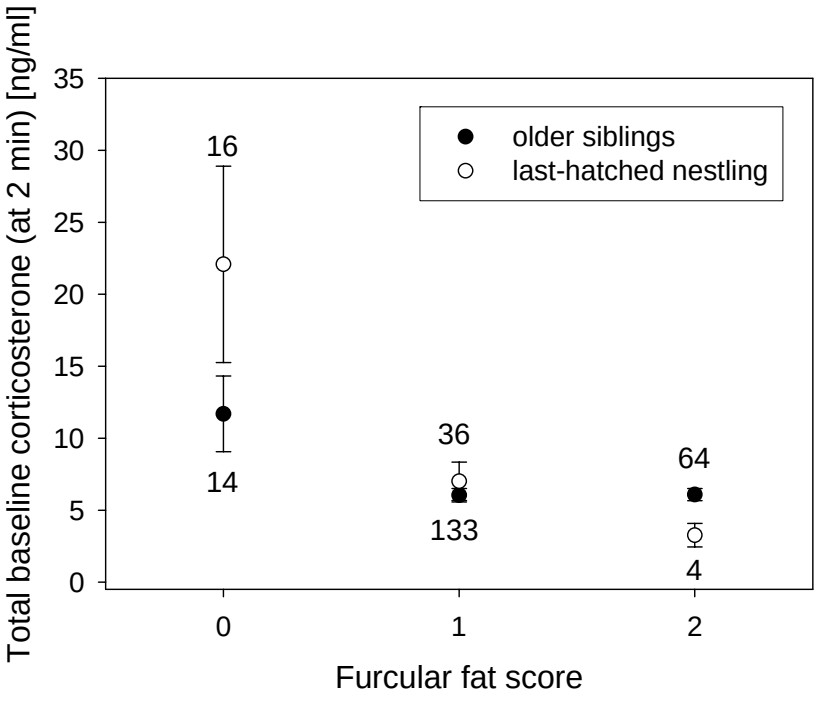

Fig. 5. Mean total baseline corticosterone (± SE) on day 10 of last-hatched nestlings and older siblings depending on furcular fat score. Total baseline corticosterone levels have been corrected for a time after capture of 2 min (the mean time after capture) according to the slope from the model given in Table 2. Numbers near error bars indicate sample sizes.

Table 4. Dependence of baseline total and free corticosterone, CBG-capacity and handling-induced (17 min after capture) corticosterone in kestrel nestlings on furcular fat score, age difference to the oldest sibling and being the last-hatched of the brood and their interactions (fixed factors in a mixed model with brood identity as random factor) for day 10 of age. Significant effects are highlighted in bold.

Nestling day 10		Baseline						Handling-induced	
		Total cort (n=267)		Free cort (n=237)		CBG cap. (n=237)		Total cort (n=233)	
Explanatory variable	d.f.	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p
Time after capture [min]	1	10.11	**0.001**	4.62	**0.032**	0.15	0.702	0.03	0.862
Furcular fat score	2	29.09	**<0.001**	25.56	**<0.001**	0.01	0.994	7.17	**0.028**
Age difference to the oldest sibling [d]	1	0.16	0.691	0.05	0.815	0.89	0.346	0.01	0.925
Last-hatched nestling of the brood	1	2.72	0.099	0.45	0.504	1.19	0.275	0.32	0.573
Furcular fat score x Age difference	2	0.26	0.878	1.44	0.486	5.63	0.060	1.63	0.442
Furcular fat score x Last-hatched nestling	2	8.35	**0.015**	1.79	0.408	0.45	0.798	0.41	0.813

hatched nestlings in good condition (furcular fat score 2, n = 4, Fig. 5) tended to have lower baseline total corticosterone levels than older siblings with the same fat score (t = 1.85, d.f. = 66, p = 0.068), contrary to last-hatched nestlings in poor condition (furcular fat score 0) which had higher baseline total corticosterone levels than older siblings in poor condition (t = -1.103, d.f. = 22.625, p = 0.282, Fig. 5), but these differences were not significant when tested post-hoc and singly.

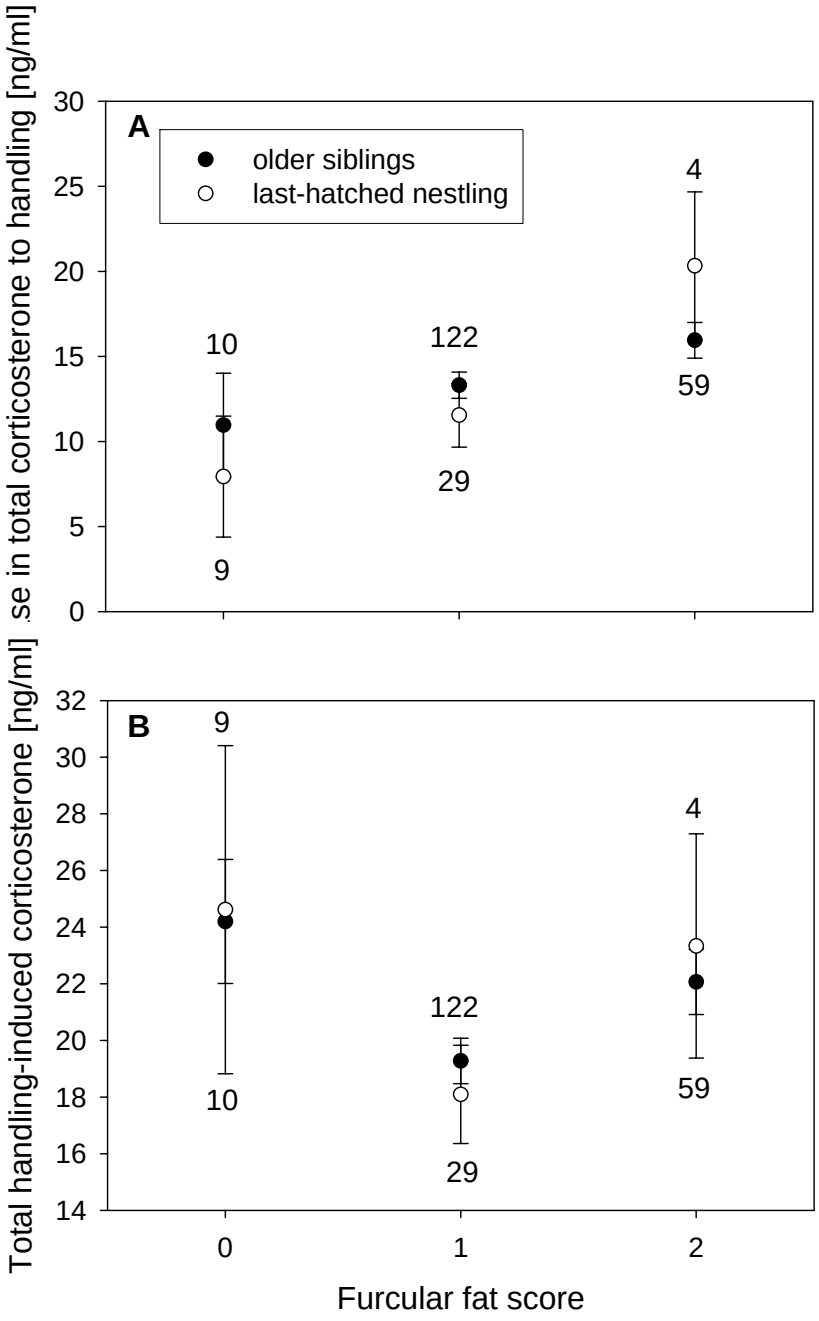

Fig. 6. (A) Increase in total corticosterone as a response to handling (difference between baseline and handling-induced total corticosterone) and (B) handling-induced total corticosterone 17 min after capture on day 10 of last-hatched nestlings and older siblings depending on furcular fat score. Numbers near error bars indicate sample sizes.

The increase in corticosterone to handling (difference between baseline and handling-induced levels) was positively related with fat stores (Wald = 11.34, d.f. = 1, p = 0.003, Fig. 6A). Because baseline levels were high in chicks in poor condition, this resulted in a U-shaped relation between absolute handling-induced corticosterone levels and furcular fat stores (Table 4, Fig. 6B), i.e. high handling-induced corticosterone levels in nestlings with furcular fat score 0 and 2 and lower handling-induced corticosterone in nestlings with furcular fat score 1. Last-hatched nestlings tended to have a smaller increase than their older siblings when in poor condition, and a larger increase when in good condition (interaction Furcular fat score x Last-hatched nestling, Wald = 5.77, d.f. = 2, p = 0.056, Fig. 6A).

On day 21, there were no significant relationships between furcular fat score and corticosterone levels (p > 0.192) and no interaction with the last-hatched nestling (p > 0.677).

3.4. Growth and corticosterone in last-hatched nestlings

Primary and body-mass growth rates of the last-hatched nestlings, after correcting for the individual nestling age, corresponded to the rates of their older siblings during the periods day 10 – 13

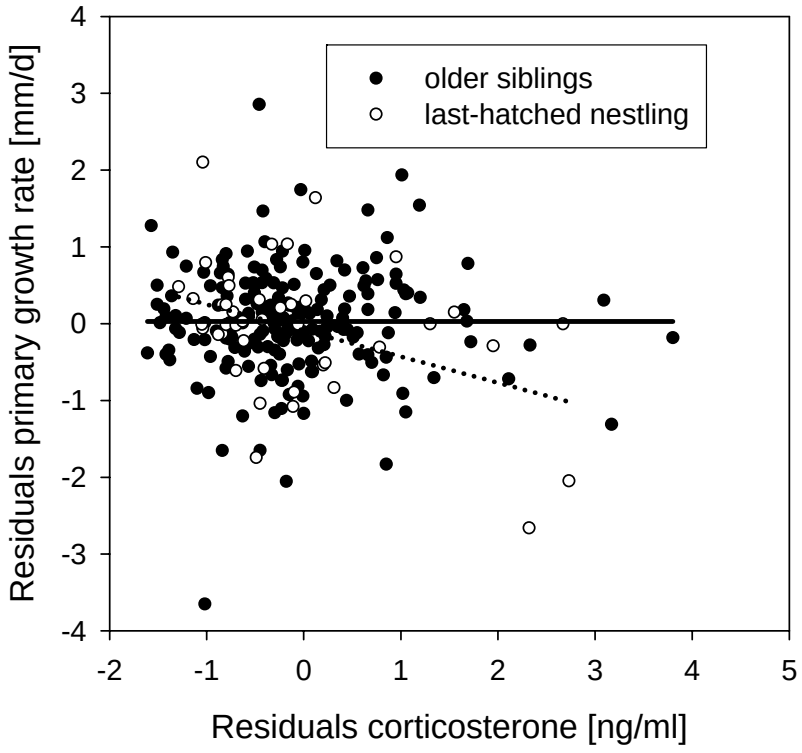

Fig. 7. Residuals of the primary growth rate from day 10 to 13 plotted against the residuals of baseline total corticosterone for last-hatched nestlings and older siblings. Residuals of the primary growth are of the relationship between primary growth and individual nestling age (mixed model analysis with brood as random factor). Residuals of baseline total corticosterone are of the relationship between baseline total corticosterone and time after capture.

Table 5. Dependence of daily primary growth rate and body mass increase rate in kestrel nestlings on individual nestling age, being the last-hatched of the brood, baseline total corticosterone (square-root transformed and corrected for time after capture) and their interaction (fixed factors in a mixed model with brood identity as random factor) from day 10 to 13 and 21 to 25 of age. Significant effects are highlighted in bold.

		Day 10-13				Day 21-25			
		Primary growth rate (n=213)		Body mass increase (n=260)		Primary growth rate (n=205)		Body mass increase (n=205)	
Explanatory variable	d.f.	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p	Wald	X^2-p
Individual nestling age [d]	1	59.75	<0.001	0.02	0.899	9.20	0.002	13.44	<0.001
Last-hatched nestling of the brood	1	0.55	0.457	0.00	0.958	2.11	0.146	1.01	0.314
Baseline total corticosterone [ng/ml] on day 21	1	3.56	0.059	0.28	0.598	5.31	0.021	2.41	0.120
Baseline total corticosterone [ng/ml] x Last-hatched nestling	3	8.01	0.005	4.06	0.044	0.00	0.973	0.68	0.409

and day 21 – 25 (Table 5), and the same was true for other growth periods (day 13 - 16, day 16 – 21; P > 0.092). However, primary and body-mass growth rates during the period 10 – 13 days depended on baseline total corticosterone levels in interaction with being the last-hatched nestling (Table 5; interaction Last-hatched nestling x Baseline corticosterone significant). In last-hatched nestlings primary growth rates were negatively related to baseline total corticosterone levels, while in older nestlings, this relationship was very shallow (Table 5, Fig. 7). When baseline corticosterone levels were low, last-hatched nestlings tended to grow faster than older siblings, while the reverse held when baseline corticosterone levels were high (Fig. 7). A similar pattern was apparent in body-mass growth (Table 5).

From day 21 to 25, nestlings with higher baseline total corticosterone levels showed a reduced primary growth rate (Table 5, effect = -0.13 ± 0.06 $mm*ml*d^{-1}*ng^{-1}$) after correcting for the individual nestling age, but there was no such relationship with body-mass growth and no interaction with the last-hatched nestlings (Table 5).

A similar analysis as given in Table 5 with free baseline corticosterone instead of total baseline corticosterone provided similar relationships.

3.3. Mortality and corticosterone

Nestling mortality (mostly starvation) occurred predominantly between day 10 and 16. Of 294 nestlings on day 10, 20 died up to day 13 and 9 more until day 16 (9.9% over 7 days), while only 3

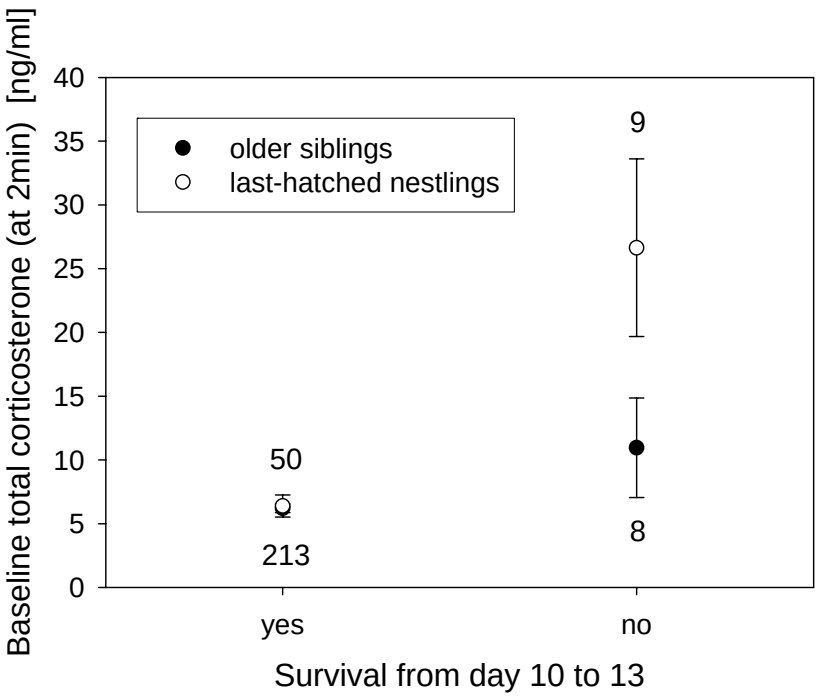

Fig. 8. Baseline total corticosterone on day 10 of last-hatched nestlings and older siblings, depending on whether they survived until day 13 or not. Baseline total corticosterone levels have been corrected for a time after capture of 2 min (the mean time after capture) according to the slope from the model given in Table 2. Numbers near error bars indicate sample sizes.

died between day 16 and 21 and 3 between day 21 and 25. 12 of the 20 nestlings which died from day 10 to 13 were the last-hatched of the brood and the remaining 8 were older siblings in those broods, where the last-hatched had died.

Nestlings that died between day 10 and 13 had clearly higher baseline total corticosterone levels on day 10 than those that reached the age of 13 days (Wald = 6.96, d.f. = 1, p = 0.008; mean ± SE: surviving 6.40 ± 0.33 ng/ml, n = 263; not surviving 24.53 ± 6.32 ng/ml, range 2.52 – 63.62 (103.40) ng/ml, n = 182; Fig. 8). A binomial mixed model analysis on survival rate between day 10 and 13 revealed, that starving nestlings had higher corticosterone levels than surviving nestlings (Wald = 6.96, d.f. = p = 0.008), last-hatched nestlings died more often than older siblings (Wald = 44.26, d.f. = 1, p < 0.001) and starving last-hatched nestlings had higher corticosterone levels than starving older siblings (Wald = 6.50, d.f. = 1, p = 0.011, Fig. 8)

Only 3 nestlings died between day 21 and 25 (surviving 8.46 ± 0.40 ng/ml, n = 248; not surviving 14.61 ± 6.03 ng/ml, range 4.05 – 24.92 ng/ml, n = 3).

A binomial mixed model analysis with free baseline corticosterone instead of total baseline corticosterone provided similar relationships.

4. Discussion

In this study, we demonstrated that last-hatched nestlings in asynchronous broods of European kestrels differed in baseline corticosterone concentrations, in the adrenocortical response to an acute stressor (handling and restraint), in body fat stores and primary growth rate from their older siblings. Most importantly, the differences between last-hatched and older nestlings in these aspects were condition-dependent and varied with food conditions. This demonstrates that the strategy of the last-hatched nestling differs from that of older nestlings, and is adapted to the prevailing food conditions much more than in older nestlings. It may switch from a catch-up strategy under good conditions to a survival strategy in bad conditions.

4.1. Corticosterone and growth of the last-hatched nestling under good conditions

Under good conditions we predicted lower baseline corticosterone levels and a higher primary growth rate in last-hatched nestlings compared with older siblings. This was confirmed on day 10. Without rain during the preceding days, free baseline corticosterone levels were significantly lower in last-hatched nestlings than in older siblings (Fig. 3). Similarly, we found almost significantly lower baseline total corticosterone levels in those few last-hatched nestlings with relatively large amounts of furcular fat stores compared with older siblings with similar fat stores (Fig. 5). This pattern corresponds with that found in captive, *ad libitum* fed American kestrels *Falco sparverius* and canaries *Serinus canaria* (Schwabl, 1999; Love et al., 2003), whose later-hatched nestlings had lower baseline corticosterone levels than first-hatched.

One function of particularly low corticosterone levels in last-hatched nestlings under good conditions could be to allow a very fast feather growth and, thus, a partial compensation of the backlog in wing length to older siblings. Corticosterone is down-regulated during moult (Romero, 2002) and elevated levels have been shown to reduce feather growth (Romero et al., 2005; Strochlic and Romero, 2008; Müller et al., 2009b). Indeed, last-hatched nestlings with low corticosterone levels tended to show a faster primary growth rate than older siblings (Fig. 7). To attain similar wing feather lengths at fledging as the siblings seems to be a crucial factor for last-hatched nestlings. Other studies also found the same rate or even faster feather growth in last-hatched nestlings compared with older siblings, partially at the expense of body mass and tarsus development (Skagen, 1987; Nilsson and Svensson, 1996; Lago et al., 2000).

Another function of low baseline corticosterone levels in later-hatched nestlings under good conditions could be to dampen a possible rise in corticosterone when conditions deteriorate (later-hatched nestlings being competitively disadvantaged) to avoid high, growth-suppressing corticosterone levels. This is confirmed by the fact that baseline total and free corticosterone levels did not vary with within-brood age (Table 2, 3), although body condition in later-hatched nestlings was worse than in first-hatched (Fig. 4). Similarly, other studies with free-living, altricial nestlings found baseline corticosterone levels independent of hatching asynchrony (Sockman and Schwabl, 2001; Blas et al., 2005). When conditions deteriorate further, however, the bad condition of starving last-hatched nestlings results in high corticosterone levels. This is consistent with a study in altricial collared dove *Streptopelia decaocto* nestlings, where last-hatched nestlings were in a worse condition and had higher baseline corticosterone than older siblings (Eraud et al., 2008).

Low baseline corticosterone levels and a high growth rate in last-hatched nestlings under good condition were only found on day 10 and not on day 21. The within-brood hierarchy in size is much stronger on day 10 than on day 21, when older nestlings normally have reached maximum body mass and the surviving later-hatched siblings are catching up. Additionally, feather growth is at its maximum rate around day 21 in the kestrel (Müller et al., 2009b) and presumably cannot be accelerated by later-hatched nestlings without restrictions on quality (Dawson et al., 2000).

4.2. Long-term food shortage and corticosterone in the last-hatched nestling

We showed that kestrel nestlings grew slower, had less body energy stores and tended to have a higher mortality in 2004 than in 2005, but brood size did not differ. This indicates that food conditions were worse in 2004 than 2005, but allowed to catch-up in body mass, but not in feather length, in the surviving nestlings until day 21.

Contrary to our prediction, kestrel nestlings did not have elevated baseline corticosterone levels in the poorer year compared with the good year; on the contrary, on day 21 they had somewhat higher baseline levels in the year with better food conditions. The lower food availability in 2004 therefore did not result in elevated baseline corticosterone levels, indicating a long-term food

shortage. This is consistent with a study in free-living black-legged kittiwake nestlings, which also did not have higher baseline corticosterone levels in years with a lower food availability (Brewer et al., 2008). In contrast to these free-living nestlings, altricial nestlings in lab studies had higher baseline corticosterone levels under experimental food restriction (Kitaysky et al., 1999; Pravosudov and Kitaysky, 2006). One explanation for this discrepancy could be that free-living adults adapt their reproduction to the prevailing food conditions (lower breeding density, reduced clutch size, higher hunting effort) or that, in asynchronous hatching species, brood size is adaptively reduced to the prevailing conditions and thus prevents elevated baseline corticosterone in free-living nestlings. In the case of our study, the presumably lower food availability in 2004 was met by a lower breeding density of the kestrels, a slower growth rate and a higher nestling mortality.

Corresponding to our prediction, the adrenocortical response to handling was stronger in 2005 with better food conditions than in 2004 on both day 10 and 21. This stronger adrenocortical response to handling in 2005 can be explained with a generally better condition in 2005 than in 2004 (faster structural and body mass growth and higher body fat stores). In accordance, the increase in corticosterone as a response to handling on day 10 positively correlated with furcular fat stores (Fig. 6), indicating, that the costs of a strong rise in corticosterone as a response to an acute stressor, associated with high energy expenditure (DuRant et al., 2008), are only sustained with a certain amount of body energy stores. This hypothesis is further supported by the higher handling-induced corticosterone levels of first-hatched nestlings in 2005 compared with 2004 (Fig. 2); first-hatched nestlings in the good year 2005 also had the largest body energy stores.

While we found marked differences in handling-induced corticosterone levels between years, last-hatched nestlings did not differ much in baseline and handling-induced corticosterone levels from older siblings, as predicted. We have no explanation for the higher handling-induced total corticosterone levels of last-hatched nestlings compared with older siblings in 2005. The higher CBG capacity in 2005 compared with 2004 on day 10 and 21, is difficult to interpret. However, free corticosterone was not different between years. In contrast to these findings, CBG capacity of barn owl *Tyto alba* nestlings was lower and baseline free corticosterone higher in a year with high food availability compared to a year with lower food availability (Almasi et al., submitted).

4.3. Corticosterone, growth and survival of the last-hatched nestling during an episodic food shortage

We found only little effect of rain on growth rates (primary growth rate between day 13 and 16). We might have missed an effect of rain on growth rates because rain periods lasted for only 1 – 3 days in the study years and rain periods rarely coincided with a particular growth measurement period. Furthermore, parents may allocate less food to themselves to buffer the effect of rain on the nestlings (Jenni-Eiermann et al., 2008). After the rain, nestlings may catch up growth to a certain extent, masking the direct effect of rain on growth.

As predicted, last-hatched nestlings had elevated baseline (total and free) corticosterone levels on day 10, if there was rainfall during the two preceding days (Fig. 3). Rain reduces the hunting success of adult kestrels (Cavé, 1968) and thus reduces food availability for their nestlings, resulting in a stronger competition for food between siblings. This is probably most effective during the middle of the nestling period, the period of brood reduction. Indeed, we found an effect of rain on feather growth only during this period. A reduced food availability affects most strongly the youngest and presumably least competitive nestlings within the brood, which at the same time have the smallest amount of fat stores as an insurance for such fluctuations in food availability. These last-hatched nestlings then change into an emergency life-history stage by increasing corticosterone secretion to adapt behaviour and metabolism, suppressing anabolic and promoting catabolic processes (Wingfield et al., 1998). Increased corticosterone has been shown to increase begging in altricial nestlings (Kitaysky et al., 2001; Loiseau et al., 2008) and generally mobilizes stored energy from body stores (e.g. reviewed in Carsia and Harvey, 2000).

Contradicting our prediction, handling-induced corticosterone levels during rainy periods were not reduced in last-hatched nestlings compared with older siblings, but elevated in younger nestlings within the brood. This may be explained by the already high baseline corticosterone levels in last-hatched nestlings in low body condition (similarly high as handling-induced levels) and the fact that corticosterone levels generally do not decrease with handling.

As predicted, wing feather growth rate was reduced if baseline corticosterone levels were elevated on day 10 and 21. This confirms the feather-growth inhibiting effect of elevated corticosterone levels found in other studies (Romero et al., 2005; Müller et al., 2009b). This relationship was modulated on day 10 in last-hatched nestlings, whose wing feather growth rate was more strongly reduced than in older siblings when corticosterone levels were high. It may be, that protein stores in last-hatched nestlings in a bad condition are few and result in a substrate deficiency for feather growth, contributing to the growth suppressing effects of corticosterone alone (which is also seen in older siblings).

Nestlings, that did not survive from day 10 to 13 or day 21 to 25, had significantly higher baseline total and free corticosterone levels on day 10 or 21, respectively. A similar pattern was found in free-living Eurasian tree creeper *Certhia familiaris* nestlings (Suorsa et al., 2003). Last-hatched nestlings that did not survive had higher corticosterone levels than older nestlings that died (Fig. 8). This is probably because last-hatched nestlings died first and were closer to starvation death than their older siblings that died later. Very high corticosterone levels close to starvation death are typical and indicate the mobilisation of the last protein to be catabolized (Cherel et al., 1988; Jenni et al., 2000).

4.4. Conclusions

In this study we showed that last-hatched nestlings in asynchronous broods of European kestrels differed from older siblings in the strategy of growth, of storing body energy stores and of dealing with short-term food restrictions. These strategies were strongly condition-dependent. It is interesting to note that brood hierarchy or within-brood age difference often was a less good predictor of the strategy adopted than being the last-hatched.

Under good conditions, last-hatched nestlings seem to allocate energy to growth, rather than storing body stores. A fast primary growth in last-hatched nestlings, also found in other studies (Nilsson and Svensson, 1996), may be facilitated by low baseline corticosterone levels, similar as during moult in adult birds (Romero, 2002). Before only found in lab studies with ad libitum fed nestlings, we found evidence for lower baseline corticosterone levels in last-hatched nestlings in free-living birds. This could represent a modulation of the hypothalamo-pituitary-axis with brood hierarchy, allowing last-hatched nestlings to catch-up with older siblings under good conditions. Future studies supplementing food in free-living nestlings could further clarify the situation.

Under conditions of short-term food shortage, last-hatched nestlings first seem to moderately increase corticosterone levels and reach only average levels, as in older siblings, because of their low baseline total corticosterone levels under good conditions. However, if bad conditions prevail, last-hatched nestlings, because of their notoriously low body energy stores, switch to a survival strategy much more rapidly and thoroughly than older siblings. Their corticosterone levels increase dramatically before starvation.

In summary we found that the adaptation of the hypothalamo-pituitary-axis to the within-brood status of the nestling (being the last-hatched) reinforces the effects of asynchronous hatching and, thus supports the brood reduction hypothesis by Lack (1954). This condition-dependent adaptation of the hypothalamo-pituitary-axis ameliorates the survival of the last-hatched nestling under good conditions (one of the extensions of the brood reduction hypothesis proposed by Pijanowski (1992)), while under bad conditions, small body energy stores (for the sake of fast growth under good conditions) accelerate starvation of the last-hatched.

Acknowledgements

We warmly thank Ueli Schaffner, Renate Wullschleger, Martin Fischer, Martin Gassner, Linda Bischoff, Christophe Bonetti, Annette Sauter, Jodok Guntern, Matthias Ernst, Lena Senn, for their help in the field work. We are grateful to Ester Pellegrini and Monika Pfunder, who performed the molecular sex determination at the Swiss Federal Institute for Forest, Snow and Landscape Research in Birmensdorf and at the Agroscope Changins-Wädenswil Research Station ACW in Wädenswil. The Federal Office of Meteorology and Climatology MeteoSwiss kindly provided the rain

data. Financial support for this study was provided by the Swiss National Science Foundation Grant 3100A0-104134 to L.J and S.J-E.

References

Almasi, B., Roulin, A., Jenni-Eiermann, S., Breuner, C.W., Jenni, L., submitted. Regulation of circulating free corticosterone and CBG capacity under different environmental conditions in altricial nestlings

Barsano, C.P., Baumann, G., 1989. Simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria; or how to calculate bound and free hormone? Endocrinology 124, 1101-1106.

Blas, J., Baos, R., Bortolotti, G.R., Marchant, T., Hiraldo, F., 2005. A multi-tier approach to identifying environmental stress in altricial nestling birds. Functional Ecology 19, 315-322.

Breuner, C.W., Orchinik, M., 2002. Plasma binding proteins as mediators of corticosteroid action in vertebrates. Journal of Endocrinology 175, 99-112.

Breuner, C.W., Orchinik, M., Hahn, T.P., Meddle, S.L., Moore, I.T., Owen-Ashley, N.T., Sperry, T.S., Wingfield, J.C., 2003. Differential mechanisms for regulation of the stress response across latitudinal gradients. American Journal of Physiology 285, R594-R600.

Brewer, J.H., O'Reilly, K.M., an Kildaw, S., Loren Buck, C., 2008. Interannual variation in the adrenal responsiveness of black-legged kittiwake chicks (*Rissa tridactyla*). General and Comparative Endocrinology 156, 361-368.

Carsia, R.V., Harvey, S., 2000. Adrenals. In: Whittow, G.C. (Ed.), Sturkie's Avian Physiology. Academic Press, San Diego, pp. 489-537.

Cavé, A.J., 1968. The breeding of the kestrel, *Falco tinnunculus* L., in the reclaimed area Oostelijk Flevoland. Netherlands Journal of Zoology 18, 313-407.

Cherel, Y., Robin, J.-P., Le Maho, Y., 1988. Physiology and biochemistry of long-term fasting in birds. Canadian Journal of Zoology 66, 159-166.

Dawson, A., Hinsley, S.A., Ferns, P.N., Bonser, H.C., Eccleston, L., 2000. Rate of moult affects feather quality: a mechanism linking current reproductive effort to future survival. Proceedings of the Royal Society B – Biological Sciences 267, 2093-2098.

DuRant, S.E., Romero, L.M., Talent, L.G., Hopkins, W.A., 2008. Effect of exogenous corticosterone on respiration in a reptile. General and Comparative Endocrinology 156, 126-133.

Eraud, C., Trouve, C., Dano, S., Chastel, O., Faivre, B., 2008. Competition for resources modulates cell-mediated immunity and stress hormone level in nestling collared doves (*Streptopelia decaocto*). General and Comparative Endocrinology 155, 542-551.

Forbes, L.S., 1993. Avian Brood Reduction and Parent-Offspring "Conflict". The American Naturalist 142, 82-117.

Fridolfsson, A.K., Ellegren, H., 1999. A simple and universal method for molecular sexing of non-ratite birds. Journal of Avian Biology 30, 116-121.

Hillstrom, L., Olsson, K., 1994. Advantages of hatching synchrony in the pied flycatcher *Ficedula hypoleuca*. Journal of Avian Biology 25, 205-214.

Husby, M., 1986. On the Adaptive Value of Brood Reduction in Birds: Experiments with the Magpie *Pica pica* . Journal of Animal Ecology 55, 75-83.

Jenni, L., Jenni-Eiermann, S., Spina, F., Schwabl, H., 2000. Regulation of protein breakdown and adrenocortical response to stress in birds during migratory flight. American Journal of Physiology 278, R1182-R1189.

Jenni-Eiermann, S., Glaus, E., Gruebler, M., Schwabl, H., Jenni, L., 2008. Glucocorticoid response to food availability in breeding barn swallows (*Hirundo rustica*). General and Comparative Endocrinology 155, 558-565.

Kaiser, A., 1993. A new multi-category classification of subcutaneous fat deposits of songbirds. Journal of Field Ornithology 64, 246-255.

Kawasaki, E.S., 1990. Sample preparation from blood, cells, and other fluids. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols - A guide to methods and applications. Academic Press, New York, pp. 146-152.

Kitaysky, A.S., Piatt, J.F., Wingfield, J.C., Romano, M., 1999. The adrenocortical stress-response of Black-legged Kittiwake chicks in relation to dietary restrictions. Journal of Comparative Physiology - B, Biochemical, Systemic, and Environmental Physiology 169, 303-310.

Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 2001. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. Behavioral Ecology 12, 619-625.

Lack, D., 1954. The natural regulation of animal numbers. Clarendon Press, Oxford.

Lago, K., Johnson, L.S., Albrecht, D.J., 2000. Growth of late-hatched, competitively disadvantaged nestling house wrens relative to their older, larger nestmates. Journal of Field Ornithology 71, 676-685.

Legge, S., 2002. Siblicide, starvation and nestling growth in the laughing kookaburra. Journal of Avian Biology 33, 159-166.

Loiseau, C., Sorci, G., Dano, S., Chastel, O., 2008. Effects of experimental increase of corticosterone levels on begging behavior, immunity and parental provisioning rate in house sparrows. General and Comparative Endocrinology 155, 101-108.

Love, O.P., Bird, D.M., Shutt, L.J., 2003. Plasma corticosterone in American kestrel siblings: Effects of age, hatching order, and hatching asynchrony. Hormones and Behavior 43, 480-488.

Magrath, R.D., 1990. Hatching asynchrony in altricial birds. Biological Reviews 65, 587-622.

Müller, C., Almasi, B., Roulin, A., Breuner, C.W., Jenni-Eiermann, S., Jenni, L., 2009a. Effects of corticosterone pellets on baseline and stress-induced corticosterone and corticosteroid-binding-globulin. General and Comparative Endocrinology 160, 59-66.

Müller, C., Jenni-Eiermann, S., Jenni, L., 2009b. Effects of a short period of elevated circulating corticosterone on postnatal growth in free-living Eurasian kestrels Falco tinnunculus. Journal of Experimental Biology 212, 1405-1412.

Müller, C., Jenni-Eiermann, S., Jenni, L., submitted. Development of the adrenocortical response to stress in Eurasian kestrel nestlings *Falco tinnunculus*: the importance of condition and brood hierarchy.

Munro, C., Stabenfeldt, G., 1984. Development of a microtitre plate enzyme immunoassay for the determination of progesterone. Journal of Endocrinology 101, 41-49.

Munro, C.J., Lasley, B.L., 1988. Non-radiometric methods for immunoassay of steroid hormones. In: Albertson, B.D., Haseltine, F.P. (Eds.), Non-radiometric assays : technology and application in polypeptide and steroid hormone detection. Alan R. Liss, Inc, cop., New York, pp. 289-329.

Newton, I., 1979. Population Ecology of Raptors. T & A D Poyser, Berkhamsted.

Nilsson, J.A., Svensson, M., 1996. Sibling Competition Affects Nestling Growth Strategies in Marsh Tits. Journal of Animal Ecology 65, 825-836.

Payne, R.W., 2003. The Guide to GenStat Release 7.1., Part 2: Statistics. VSN International, Oxford.

Pijanowski, B.C., 1992. A Revision of Lack's Brood Reduction Hypothesis. The American Naturalist 139, 1270-1292.

Pravosudov, V.V., Kitaysky, A.S., 2006. Effects of nutritional restrictions during post-hatching development on adrenocortical function in western scrub-jays (*Aphelocoma californica*). General and Comparative Endocrinology 145, 25-31.

Ricklefs, R.E., 1965. Brood reduction in the curve-billed trasher. Condor 67, 505-510.

Romero, L.M., 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. General and Comparative Endocrinology 128, 1-24.

Romero, L.M., Strochlic, D., Wingfield, J.C., 2005. Corticosterone inhibits feather growth: Potential mechanism explaining seasonal down regulation of corticosterone during molt. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 142, 65-73.

Schwabl, H., 1999. Developmental changes and among-sibling variation of corticosterone levels in an altricial avian species. General and Comparative Endocrinology 116, 403-408.

Skagen, S.K., 1987. Hatching Asynchrony in American Goldfinches: An Experimental Study. Ecology 68, 1747-1759.

Sockman, K.W., Schwabl, H., 2001. Plasma corticosterone in nestling American kestrels: Effects of age, handling stress, yolk androgens, and body condition. General and Comparative Endocrinology 122, 205-212.

Stenning, M.J., 1996. Hatching asynchrony, brood reduction and other rapidly reproducing hypotheses. Trends in Ecology and Evolution 11, 243-246.

Strochlic, D.E., Romero, L.M., 2008. The effects of chronic psychological and physical stress on feather replacement in European starlings (*Sturnus vulgaris*). Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 149, 68-79.

Suorsa, P., Huhta, E., Nikula, A., Nikinmaa, M., Jäntti, A., Helle, H., Hakkarainen, H., 2003. Forest management is associated with physiological stress in an old-growth forest passerine. Proceedings of the Royal Society of London - B: Biological Sciences 270, 963-969.

Tarlow, E.M., Wikelski, M., Anderson, D.J., 2001. Hormonal correlates of siblicide in galapagos Nazca boobies. Hormones and Behavior 40, 14-20.

Thompson, R., Welham, S.J., 2003. REML analysis of mixed models. In: Payne, R.W. (Ed.), The Guide to GenStat Release 7.1., Part 2: Statistics VSN International, Oxford, pp. 513-607.

Valkama, J., Korpimäki, E., Holm, A., Hakkarainen, H., 2002. Hatching asynchrony and brood reduction in Tengmalm's owl *Aegolius funereus*: the role of temporal and spatial variation in food abundance. Oecologia 133, 334-341.

Wada, H., Hahn, T.P., Breuner, C.W., 2007. Development of stress reactivity in white-crowned sparrow nestlings: Total corticosterone response increases with age, while free corticosterone response remains low. General and Comparative Endocrinology 150, 405-413.

Wiehn, J., Ilmonen, P., Korpimäki, E., Pahkala, M., Wiebe, K.L., 2000. Hatching asynchrony in the Eurasian kestrel *Falco tinnunculus*: An experimental test of the brood reduction hypothesis. Journal of Animal Ecology 69, 85-95.

Wingfield, J.C., Maney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M., Richardson, R.D., 1998. Ecological Bases of Hormone-Behavior Interactions: The "Emergency Life History Stage". American Zoologist 38, 191-206.

Synthesis

Synthesis

The aim of this thesis was to examine the significance and role of corticosterone during postnatal development in free-living birds. With its steering role in energy allocation, corticosterone supports an organism to get over threatening situations. But concurrently future prospects possibly deteriorate because of negative impacts of corticosterone on growth and development and potential deleterious effects on the phenotype.

To understand the role of corticosterone, it is important to know, 1) which factors of the natural environment (food availability, hatching order) or of the actual body condition have the potential to increase circulating baseline corticosterone, 2) which effects elevated circulating corticosterone has on growth and survival, 3) whether the dynamics of corticosterone are modulated with age or condition during the postnatal phase. We further investigated, whether circulating corticosterone is modulated by a varying CBG capacity, resulting in different patterns in free corticosterone.

While many studies have investigated stressors provoking a rise in corticosterone and the effects of corticosterone in the lab (possibly including pharmacological levels), studies in free-living birds interacting with a varying environment are especially valuable. In the field, birds have a range of body conditions and live under different environmental conditions. The animals are exposed to natural selective pressures and potential fitness consequences can be studied if it is possible to track the animals.

Baseline corticosterone, condition and hatching asynchrony

In free-living, asynchronously hatching Eurasian kestrel nestlings exposed to yearly and weather-dependent fluctuating food availability, we found a strong condition-dependence of baseline corticosterone levels. While baseline corticosterone levels were generally low, they were clearly elevated in birds in low condition and in last-hatched nestlings under reduced food availability with rain. The low baseline corticosterone levels allow optimal growth and development. However, when food is scarce and no fat reserves remain to safeguard maintenance, corticosterone levels rise to promote metabolization of protein reserves and suppress growth. Facing threatening starvation death, an interference with the morphological phenotype is unavoidable.

Considering that fat stores explained a substantial part of the variation in baseline corticosterone, it is recommendable, to always assess subcutaneous fat stores in lab and field studies on corticosterone, a fast, easily applied and non-invasive method. This allows to consider interactions of body condition with the investigated parameter (many effects of corticosterone have been shown to be condition dependent; e.g. Astheimer et al., 1992; Loiseau et al., 2008; Roulin et al., 2008) or to correct for a condition-dependent rise in corticosterone.

The condition-dependence of baseline corticosterone levels was modulated by hatching asynchrony. Last-hatched nestlings showed lower baseline corticosterone levels when in a good

condition and higher baseline corticosterone levels when in a bad condition than first-hatched ones. We have two possible explanations for lower corticosterone levels in later-hatched nestlings under good conditions: a) They could dampen a condition-dependent rise in baseline corticosterone in later-hatched nestlings, resulting in similarly high corticosterone levels as in older siblings. This is confirmed by an independence of baseline corticosterone levels on hatching asynchrony under average conditions (overall mean). b) They could enable compensatory feather growth in last-hatched nestlings to catch up with older siblings. This could be part of an "all-or-nothing"-strategy of the last-hatched nestlings, which are often the ones sacrificed when the broods needs to be reduced. Last-hatched nestlings would refrain from fat reserves and invest in structural growth for a synchronous fledging with siblings under good conditions, but under bad conditions they would starve quickly. Such a strategy was confirmed by a faster primary growth in last-hatched nestlings under good conditions, lower fat reserves in last-hatched nestlings and high corticosterone levels and considerable mortality in last-hatched nestlings after a short-term reduction in food-availability caused by rain.

The condition-dependent modulation in baseline corticosterone levels and fat stores in last-hatched nestlings gives support to the brood reduction hypothesis. Under good conditions the performance of the last-hatched nestling is ameliorated, while under bad conditions the last-hatched nestlings quickly starves, presumably increasing the fledging success of the older siblings.

Free corticosterone levels correlated in most cases with total corticosterone, indicating no important role of CBG capacity in regulating total corticosterone in this study. Therefore the observed modulations were via corticosterone release and not via buffering CBG.

The mechanism by which hatching-order dependent corticosterone levels arise is not known. One possibility is the deposition of maternal androgens or glucocorticoids in the egg yolk, varying with laying sequence, which have been shown to have organizational effects on the HPA axis (Sockman and Schwabl, 2000; Sockman and Schwabl, 2001; Love et al., 2008). Another mechanism would consist of an individual modulation of the HPA axis by nestlings recognising that they are the last-hatched. The two mechanisms differ in important aspects. In the first, the mother prepares nestlings so that under good conditions the reproductive success is maximised. The second mechanism is based on the notion that individuals try to optimize their situation.

Generally, these modulations of baseline corticosterone with condition and hatching asynchrony indicate strong selective pressures for low baseline corticosterone in free-living altricial nestlings. A condition-dependent chronic elevation in corticosterone is linked with high nestling mortality by starvation.

Effects of corticosterone on growth and development

To reveal the detailed effects of baseline corticosterone on growth in a natural context, we conducted an experimental and an observational study. In the experimental approach with artificially

elevated corticosterone levels (within the range of handling-induced levels), we found clear suppressing effects to different degrees on feather and bone growth and body mass increase, indicating a steering role for corticosterone in growth allocation. The lag in growth was only partly compensated until fledging. Tarsus and hand length presumably remained shorter for life and wing-feathers shorter until the next moult. Stressors therefore can shape the phenotype via elevated corticosterone levels. However, considering our observational approach on birds under varying environmental conditions, we only rarely found individuals with baseline corticosterone similarly high as handling-induced levels, all in a very bad condition, presumably under severe nutritional stress. Those individuals usually starved until the next nest visit. Therefore, individuals suffering from a serious encroachment on morphology presumably often do not survive the nestling stage. The rare occurrence of high baseline levels and the high mortality in those nestlings to the next nest visit were probably also the reason, why we could not confirm similar growth suppressing effects of corticosterone in the observational study as in the experimental study.

Lab studies have demonstrated negative long-term effects of an elevation in baseline corticosterone during postnatal development in altricial nestlings on cognition (song performance and learning; Spencer et al., 2003; Kitaysky et al., 2003). By manipulating corticosterone levels in free-living altricial nestlings without nutritional restriction, we could now demonstrate, that corticosterone also plays a steering role in growth allocation to different structures, thereby shaping morphological traits of the phenotype. These clear effects of an elevation in corticosterone on growth and development select for low baseline corticosterone levels during postnatal development.

Acute response in corticosterone to a threat

The role of corticosterone in response to acute threats was examined in two different ways. The acute adrenocortical responses to both threats were modulated with age during the postnatal stage. While an acute response to the presence of humans (perceived as a predator) at the nest was absent in the first half of the nestling stage, when nestlings are not able to defend themselves against nest intruders, a stronger rise in corticosterone occurred in the second half of the nestling stage, when nestlings show an extended defence behaviour against nest intruders. This fight response with defence capabilities is therefore sustained by a rise in corticosterone. Similarly, the handling-induced corticosterone levels increased from the first to the second half of the nestling stage. This confirmed the developmental hypothesis, which predicts, that the adrenocortical response to handling increases with age in altricial nestlings. While in the first half of the nestling stage the benefits of a strong adrenocortical response to an acute threat are limited and the costs (growth suppression) are high, the benefits of a clear response in corticosterone seem to outweigh the costs in the second half of the nestling stage.

Besides the dependence on age, the acute adrenocortical response to handling was modulated by the amount of fat stores. Nestlings with a fair amount of fat reserves (being in a good condition), showed a stronger increase in corticosterone to handling than those without or with few

fat stores. A strong increase in corticosterone to an acute stressor enables a stronger behavioural and physiological response and presumably ameliorates the prospects of nestlings in good condition, to cope with the dangerous situation.

The increase in corticosterone to an acute stressor (difference between baseline and handling-induced corticosterone levels) proved to be a good measure to understand the relationship between baseline and handling-induced levels and showed a nice relationship with subcutaneous fat stores.

The acute response in corticosterone to a threat was therefore modulated with age and body condition and presumably optimized to the prevailing external and internal conditions. While in baseline corticosterone an elevation during postnatal development is avoided, a short-term acute rise in corticosterone enabling an appropriate behavioural and physiological adaptation to an acute stressor seems to be favoured.

Perspectives

By artificially elevating corticosterone in free-living nestlings, we found clear growth suppressing effects of corticosterone resulting in a lag in morphological parameters compared with control siblings, which could only partly be compensated until fledging. The performance of these phenotypes, shaped by a simulated stress situation during postnatal development was followed after fledging (data not presented in this thesis). We assessed the behavioural development of fledglings (predator avoidance, learning of hunting), to reveal potential negative effects of postnatal stress on cognition, as found by Kitaysky et al. (2003) and survival until independence. The most valuable measure of long-term effects of stress on the fitness of an individual would be survival during the first year of life and reproductive performance. In the case of the Eurasian kestrel, it unfortunately was not possible to obtain these data, because this species shows an extended dispersal over large distances not known before. This prevented us from assessing the survival of independent juveniles during the months after family break-up and of reproductive success during the following years. In future studies, evaluation of survival and reproduction, e.g. in resident species, would give valuable insights into the fitness consequences of postnatal stress.

References

Astheimer, L.B., Buttemer, W.A., Wingfield, J.C., 1992. Interactions of corticosterone with feeding, activity and metabolism in passerine birds. Ornis Scandinavica 23, 355-365.

Kitaysky, A.S., Kitaiskaia, E.V., Piatt, J.F., Wingfield, J.C., 2003. Benefits and costs of increased levels of corticosterone in seabird chicks. Hormones and Behavior 43, 140-149.

Loiseau, C., Fellous, S., Haussy, C., Chastel, O., Sorci, G., 2008. Condition-dependent effects of corticosterone on a carotenoid-based begging signal in house sparrows. Hormones and Behavior 53, 266-273.

Love, O.P., Wynne-Edwards, K.E., Bond, L., Williams, T.D., 2008. Determinants of within- and among-clutch variation in yolk corticosterone in the European starling. Hormones and Behavior 53, 104-111.

Roulin, A., Almasi, B., Rossi-Pedruzzi, A., Ducrest, A.L., Wakamatsu, K., Miksik, I., Blount, J.D., Jenni-Eiermann, S., Jenni, L., 2008. Corticosterone mediates the condition-dependent component of melanin-based coloration. Animal Behaviour 75, 1351-1358.

Sockman, K.W., Schwabl, H., 2000. Yolk androgens reduce offspring survival. Proceedings of the Royal Society - B: Biological sciences 267, 1451-1456.

Sockman, K.W., Schwabl, H., 2001. Plasma corticosterone in nestling American kestrels: Effects of age, handling stress, yolk androgens, and body condition. General and Comparative Endocrinology 122, 205-212.

Spencer, K.A., Buchanan, K.L., Goldsmith, A.R., Catchpole, C.K., 2003. Song as an honest signal of developmental stress in the zebra finch (*Taeniopygia guttata*). Hormones and Behavior 44, 132-139.

Acknowlegdements

Acknowlegdements

First of all I would like to warmly thank Lukas Jenni for his support in all phases of the PhD. His profound knowledge in ornithology, his scientific and statistical procedures and handling of birds were always exemplary for me. He developed the main research questions of this PhD, helped with on-coming problems in the field, helped with structuring of the chapters and greatly improved the manuscripts. Constructive discussions were always very helpful, and I like the way he gives ideas and impulses to solve problems. Together with Susi Jenni-Eiermann he received a grant from the Swiss National Science Foundation, which enabled this project. I'm very grateful to Susi Jenni-Eiermann for her support and the introduction into the determination of CBG and for many valuable discussions concerning lab and other subjects. I appreciated much to have the opportunity to attend many international conferences.

Many thanks to Lukas Keller for giving me the opportunity to make my PhD in his group at the Zoological Museum of the University of Zurich. A special thank goes to Bettina Almasi; it was nice to work together in the same project and exchange experiences and ideas and to get valuable comments on manuscripts. I warmly thank Marc Kéry and Michael Schaub for statistical advice and valuable discussions and comments. Beat Naef-Daenzer introduced me into the technique of telemetry, provided material and searched by plane dispersing juveniles. Martin Grüebler also made a search by plane and gave valuable comments on manuscripts. I would like to thank both of them very much for their support.

Renate Wullschleger, Martin Fischer, Martin Gassner, Linda Bischoff, Adrian Baumann, Maria Nuber, Christophe Bonetti, Annette Sauter, Jodok Guntern, Matthias Ernst and Lena Senn helped in the field controlling nest boxes, taking blood samples, measuring nestlings, and tracking and observing fledglings and independent juvenile kestrels. I warmly thank them for getting through long and hard field days, accepting scratches of defending nestlings and sharing nice experiences with the sweet and very bright kestrel nestlings and inquisitive fledglings.

A very special and warm thank goes to Ueli Schaffner for his huge and untiring effort in our field work. A substantial part of the field work was conducted with nestlings of the nest boxes attended by him and he introduced us to the farmers and other land owners. He energetically helped with nest box controls and measuring nestlings and installed all video cameras. His craft skills were very valuable for us. Kurt Mohler, Alois Bächli and Willy Städler let us also work with nestlings of their nest boxes and introduced us to farmers and land owners, I'm very grateful to them. Many thanks to all the farmers and land owners who gave us access to the nest boxes and sometimes provided us with coffee or cherries. Hans and Rita Rickenbacher provided the flat in Oltingen BL during the field work. Marcel Amsler performed two search flights for dispersing juveniles, I thank him very much for this valuable help.

I thank Ester Pellegrini and Monika Pfunder very much, they performed the molecular sex determination of kestrel nestlings at the Swiss Federal Institute for Forest, Snow and Landscape Research in Birmensdorf with permission and support of Felix Gugerli, and at the Agroscope

Changins-Wädenswil Research Station ACW in Wädenswil. I'm grateful to Peter Sandmeier for advice concerning pellet implantation and the Veterinärmedizinisches Labor of the University of Zurich and especially Elisabeth Rogg for introducing me into the differentiation of avian leukocytes. Ruth Widmer kindly helped to obtain a chicken plasma pool for the CBG assays from her hens. The Federal Office of Meteorology and Climatology MeteoSwiss kindly provided the rain data. Gilbert Klaus helped with medical care on a nestling which had an accident.

Guido Häfliger introduced me into the video hardware and software and gave support, Niklaus Zbinden gave many helpful advices, Paul Albisser supported us if we had problems with cars during the field work, Irene Fehr and Barbara Trösch provided administrative support, Dieter Peter helped with some technical problems, Vreni Mattmann cared for a kestrel which had an accident, and various other colleagues of the Swiss Ornithological Institute helped in some way. I thank all of them very much. I'm grateful to Karen Falsone and Lis Räber, who helped with the evaluation of video data of nestlings, and Marco Thoma, Eva Gallmann and Jérôme Duplain, who helped with data input of fledgling behaviour and fault bars.

I'm grateful to my parents who supported my studies in biology and warmly thank my sister Fabienne and my friends for supporting and accompanying me during intensive and exciting times.

This research was founded by the Swiss National Science Foundation (Grant 3100A0-104134 to L. Jenni and S. Jenni-Eiermann) and received financial support from the Swiss Ornithological Institute.

Curriculum vitae

Curriculum vitae

Claudia MÜLLER

Born 22th October 1977

Citizen of Unterbözberg AG, Switzerland

2004 – 2009	PhD at the Swiss Ornithological Institute and the Zoological Museum of the University of Zurich "Costs and benefits of a stress response on postnatal development in the kestrel *Falco tinnunculus*" (Supervisors: PD Dr. Lukas Jenni, Swiss Ornithological Institute, and Prof. Dr. Lukas F. Keller, Zoological Museum, University of Zurich)
2003	Scientific collaborator at the Swiss Ornithological Institute following up the work on influence of habitat quality on the stress hormone corticosterone in breeding blue tits
2001 – 2002	Diploma thesis at Swiss Ornithological Institute and the Zoological Institute of the University of Zurich "Influence of habitat quality on the stress hormone corticosterone in breeding blue tits *Parus caeruleus*" (Supervisors: PD Dr. Lukas Jenni, Swiss Ornithological Institute, and Prof. Dr. Heinz-Ulrich Reyer, Zoological Institute of the University of Zurich)
1997 – 2003	Studies in Biology (Zoology, Systematic Botany and Geobotany) at the University of Zurich
1993 – 1997	Neue Kantonsschule Aarau, Maturität Typus Pädaogogisch-Soziales Gymnasium

Publications

Müller, C., Jenni-Eiermann, S., Jenni, L., 2009. Effects of a short period of elevated circulating corticosterone on postnatal growth in free-living Eurasian kestrels *Falco tinnunculus*. Journal of Experimental Biology 212: 1405-1412.

Müller, C., Almasi, B., Roulin, A., Breuner, C.W., Jenni-Eiermann, S., Jenni, L., 2009. Effects of corticosterone pellets on baseline and stress-induced corticosterone and corticosteroid-binding-globulin. General and Comparative Endocrinology 160: 59-66.

Alcaide, M., Serrano, D., Negro, J.J., Tella, J.L., Laaksonen, T., Müller, C., Gal, A., Korpimäki, E., 2009. Population fragmentation leads to isolation by distance but not genetic impoverishment in the philopatric Lesser Kestrel: a comparison with the widespread and sympatric Eurasian Kestrel. Heredity 102: 190-198.

Müller, C., Jenni-Eiermann, S., Blondel, J., Perret, P., Caro, S. P., Lambrechts, M., Jenni, L., 2007. Circulating corticosterone levels in breeding blue tits *Parus caeruleus* differ between island and mainland populations and between habitats. General and Comparative Endocrinology 154: 128–136.

Müller, C., Jenni-Eiermann, S., Blondel, J., Perret, P., Caro, S. P., Lambrechts, M., Jenni, L. 2006. Effect of human presence and handling on circulating corticosterone levels in breeding blue tits (*Parus caeruleus*). General and Comparative Endocrinology 148: 163–171.

Müller, C., Holzgang, O., 2005. Schläfer auf dem Bözberg. Aargauische Naturforschende Gesellschaft Mitteillungen 36: 149-158.

Conference contributions

International Symposium on Avian Endocrinology, Leuven, Belgium, 11-15 July 2008 (oral)

6th European Ornithologists' Union conference, Vienna, Austria, 24-29 August 2007 (oral)

Biology07 - The Annual Meeting of the Swiss Zoological, Botanical and Mycological Societies, ETH Zurich, Switzerland, 15-16 February 2007 (oral)

ESF/NSF/NSERC Meeting, Coping with environmental change: integrating avian ecology and endocrinology, University of Glasgow, United Kingdom, 17-19 November 2006 (oral)

24th International Ornithological Congress, Hamburg, Germany, 13-19 August 2006 (poster)

137. Jahresversammlung der Deutschen Ornithologen-Gesellschaft, Kiel, Germany, 29 September – 4 October 2004 (oral)

Advanced training

Graduate course in Field endocrinology: Mechanisms for life cycles, with John C. Wingfield and Marilyn Ramenofsky, Nordens Ark, Sweden, 15-19 August, 2005